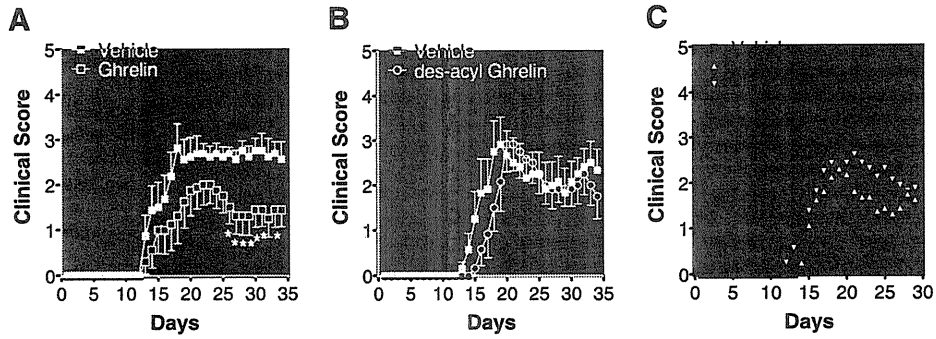


**FIGURE 1.** Effect of ghrelin on actively induced EAE. EAE was induced in female B6 mice ($n$ = 8 in each group of the three experiments) by immunization with $MOG_{35-55}$. *A*, The mice were treated every other day starting at the day of immunization with 5 μg/kg ghrelin, while controls were administrated with the vehicle, 0.9% saline, alone. *B*, The mice were injected from day 1 every other day with 5 μg/kg des-acyl ghrelin, whereas controls were subjected to 0.9% saline injections. *C*, Following an alternative protocol, mice were treated from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were treated every day with 0.9% saline injections. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.05$; Mann-Whitney $U$ test).

FACSAria (BD Biosciences). The total RNA was extracted from the isolated cells and was subjected to reverse transcription and real-time PCR.

### *In vitro effect of ghrelin on microglia cells treated with LPS*

Mononuclear cells were prepared from brains of untreated non-EAE mice incubated with Liberase Blendzyme 3 and DNase I as described above and were isolated on 40%–80% discontinuous Percoll gradients. Isolated cells were suspended in DMEM supplemented with 10% FCS and cultured in 96-well flat bottom plates at $2 \times 10^5$/well in the presence of ghrelin ($10^{-6}$ M) overnight and later stimulated with LPS at different doses (0.01, 0.1 μg/ml). After 5 h of incubation at 37°C, supernatants were collected and the levels of TNF-α were detected by using a sandwich ELISA.

### *Statistical analysis*

The differences in the clinical score between ghrelin-, des-acyl ghrelin-, and sham-treated groups were analyzed by the nonparametric Mann-Whitney $U$ test. FACS analysis, real-time PCR, ELISA, and proliferation data were subjected to two-way ANOVA. In case of significant differences, a Fisher post hoc test was applied. Probability values of <0.05 were considered as statistically significant.

## Results

### *Ghrelin inhibits EAE*

To explore the modulatory effects of ghrelin on inflammatory demyelinating diseases, we employed a model of EAE actively induced in B6 mice with $MOG_{35-55}$. Although classical forms of EAE are typically characterized by acute paralysis followed by complete recovery, this EAE model shows persistent paralysis with partial recovery as a reflection of persistent inflammatory demyelination in the CNS (21, 22). In the first series of experiments, we injected 0.5, 5, or 50 μg/kg ghrelin to the mice every other day from day 1 to 35 postimmunization, while the control mice were injected with 0.9% saline. The results showed that the continuous injections of 5 μg/kg ghrelin suppressed most efficiently the clinical signs of EAE (Fig. 1*A*), whereas a lower (0.5 μg/kg) or a higher dose (50 μg/kg) showed only a marginal effect (data not shown). The treatment with 5 μg/kg ghrelin did not significantly alter either the onset or peak score of EAE. However, significant differences were noted in mean clinical score after day 25 postimmunization between the ghrelin-treated and the control mice (Fig. 1*A*).

Moreover, the effect of ghrelin on EAE was specific as des-acyl ghrelin, an acyl-modified ghrelin, which lacks the $n$-octanoic acid on the third serine, and consequently its binding ability to GHS-R (7) (Table I) had no modulatory effect on EAE at any concentration examined (Fig. 1*B* and Table II). Thus, the discrepant results obtained with ghrelin and des-acyl ghrelin indicate that ghrelin treatment would ameliorate the clinical course of EAE via activation of the GHS-R.

To further characterize the effects of ghrelin on EAE, we next examined if treatment lasting for a shorter duration may also be immunomodulatory in vivo. We injected 5 μg/kg ghrelin every day from day 1 to 10 postimmunization (roughly corresponding to the induction phase) or from day 11 to 20 (roughly corresponding to the effector phase). As shown in Fig. 1*C*, both protocols showed similar levels of disease suppression, although it was less notable than the continuous treatment from day 1 to 35 (Table II).

### *Ghrelin does not influence cellular infiltration into CNS*

In the previous results on prophylactic or therapeutic treatment of EAE, clinical suppression of EAE was generally associated with a significant reduction of cellular infiltration in the CNS (23). To clarify if histological manifestation of EAE is also suppressed by ghrelin treatment, we treated $MOG_{35-55}$-immunized B6 mice with 5 μg/kg ghrelin or 0.9% saline every other day and prepared sections of spinal cords at the peak of disease (day 17 after immunization) (Fig. 2). Clinical signs were milder in the ghrelin-treated mice compared with saline-treated ones. However, histology of the spinal cord sections with H&E staining revealed equivalent levels of cellular infiltration in ghrelin- and saline-treated mice. To confirm this, we isolated mononuclear cells from spinal cords of the

Table II. *Clinical scores of EAE treated with ghrelin or des-acyl ghrelin following different treatment protocols*[a]

| Treatment | Incidence | Mean Day of Onset ± SEM | Mean Maximal Score ± SEM | Mean Cumulative Score ± SEM |
|---|---|---|---|---|
| Vehicle[b] | 8/8 | 16.38 ± 1.13 | 3.75 ± 0.33 | 55.44 ± 7.14 |
| Ghrelin[b] | 7/8 | 17.86 ± 1.30 | 3.29 ± 0.33 | 36.71 ± 9.99 |
| Vehicle[b] | 6/8 | 18.83 ± 2.55 | 3.67 ± 0.40 | 49.33 ± 12.99 |
| Des-acyl ghrelin[b] | 6/8 | 18.00 ± 0.71 | 3.80 ± 0.44 | 49.05 ± 8.09 |
| Vehicle[c] | 7/8 | 15.14 ± 0.51 | 4.43 ± 0.07 | 50.43 ± 3.10 |
| Ghrelin (1–10)[c] | 6/8 | 16.00 ± 0.73 | 3.17 ± 0.53 | 34.00 ± 7.25 |
| Ghrelin (11–20)[c] | 7/8 | 16.29 ± 1.25 | 3.50 ± 0.45 | 38.72 ± 8.79 |

[a] The table shows the results of three separate experiments ($n$ = 8 mice in each group of the three experiments).

[b] After induction of EAE with $MOG_{35-55}$, mice were treated in two different experiments following the standard protocol of every other day s.c. treatment with 5 μg/kg ghrelin or 5 μg/kg des-acyl ghrelin. The controls were injected with 0.9% saline (vehicle).

[c] Following an alternative protocol, we treated the mice from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9 % saline, while controls were injected every day with 0.9% saline only. Data represent mean ± SEM.

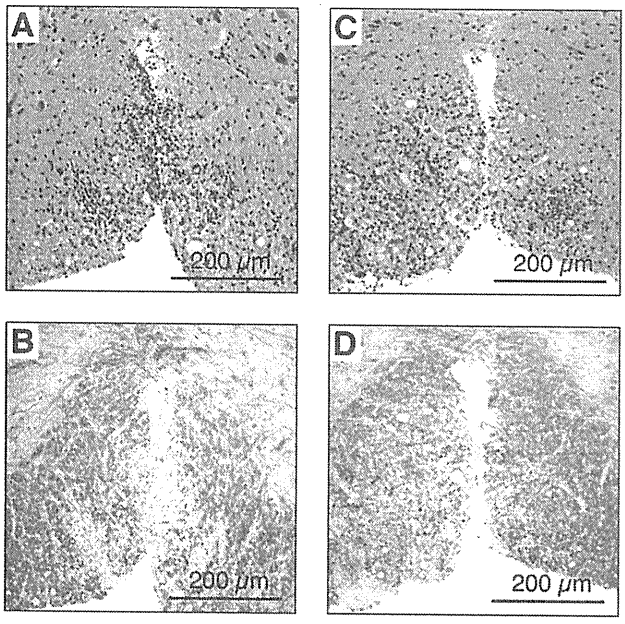


**FIGURE 2.** Histopathological assessment of the spinal cord of EAE mice. Spinal cords from EAE mice ($n$ = 5/group) were removed on day 17 postimmunization as described in *Material and Methods*. The spinal cord sections from sham- (*A* and *B*) and ghrelin-treated (*C* and *D*) mice were stained in with H&E in the upper panels or Luxol fast blue in the lower ones. Representative sections are shown.

mice at the peak of disease and enumerated the number of the lymphoid cells. Notably, the total cell number was slightly elevated in the ghrelin-treated mice ($1.40 \times 10^6$/mouse) compared with the saline-treated mice ($1.05 \times 10^6$/mouse). To further analyze the effects of ghrelin on the formation of CNS inflammation, we evaluated the cellular composition of the CNS-derived lymphocytes by using FACS. Although there was a trend that $CD4^+$ and $CD8^+$ T cell numbers are increased in the lesions of ghrelin-treated mice as compared with saline-treated mice (Fig. 3*A*), it did not reach the level of statistic significance. It was also noted that ghrelin treatment did not alter the number of NK cells

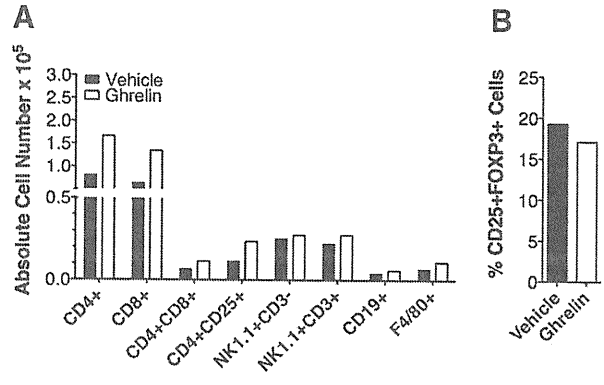


**FIGURE 3.** Quantification of spinal cord cellular infiltrates by flow cytometry. *A*, The cells were isolated from spinal cords of ghrelin- and sham-treated mice on day 17 postimmunization and subjected to flow cytometer analysis as described in *Materials and Methods*. Data are representative of two independent experiments and presented as absolute cell number ($n$ = 8 mice/group in each experiment). *B*, The proportion of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cord mononuclear cells was analyzed by flow cytometry 20 days after immunization. Data represent two independent experiments ($n$ = 5).

Table III. *Cytokine production and proliferation of $MOG_{35-55}$-specific T cells after ghrelin treatment*[a]

| Treatment | CPM ± SEM | Cytokine Production (pg/ml) INF-γ ± SEM | IL-17 ± SEM | IL-4 ± SEM |
|---|---|---|---|---|
| Vehicle | 47,590 ± 10,988 | 2,087 ± 487 | 820 ± 211 | ND |
| Ghrelin | 36,663 ± 9,058 | 2,883 ± 615 | 674 ± 148 | ND |

[a] Mice were immunized with $MOG_{35-55}$ and treated with 5 μg/kg ghrelin or 0.9% saline everyday from day 1 to 10 ($n$ = 3/group). Popliteal and inguinal LN cells were harvested on day 11 after immunization and stimulated with 10 μg/ml $MOG_{35-55}$. CPM marks the proliferative response to $MOG_{35-55}$. The cytokines were measured in the supernatant by sandwich ELISA after 72 h of stimulation. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. ND, Not detectable.

($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), B cells ($CD19^+$), or macrophages ($F4/80^+$) in the spinal cord lesions. The proportions of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cords were not altered in ghrelin-treated mice (Fig. 3*B*). In parallel, we also examined the composition of lymphoid cells obtained from spleen, LN, and thymus. Again, we could not reveal any significant change in the subsets of lymphocytes in ghrelin-treated mice (data not shown). Concordant with the histological findings, these data imply that ghrelin did not ameliorate clinical EAE by reducing the numbers of inflammatory cells in the CNS, but rather by regulating the inflammatory potential of the CNS infiltrates.

### *Ghrelin does not inhibit the induction of $MOG_{35-55}$-reactive T cells*

To elucidate the immunomodulatory mechanism of ghrelin, we examined the cytokine production and proliferative response of draining LN cells to $MOG_{35-55}$ that were obtained from $MOG_{35-55}$-sensitized mice treated for 10 days every day with ghrelin or saline. The LN cells were collected on day 11 after immunization and stimulated with $MOG_{35-55}$ in vitro. Accordingly, we harvested the supernatant and measured the levels of IFN-γ, IL-17, and IL-4 by using ELISA. Although the IL-4 concentration was under the detection level, IFN-γ and IL-17 could be detected in the $MOG_{35-55}$-stimulated culture supernatant (Table III). There was no significant difference in the level of IFN-γ and IL-17 when we compared ghrelin-treated and saline-treated groups. Furthermore, ghrelin-treated mice did not differ from saline-treated mice in the proliferative response of the draining LN cells to $MOG_{35-55}$. We also examined the frequency of $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells in the lymph nodes and spleens using flow cytometry and did not find significant differences between ghrelin-treated and saline-treated mice (data not shown). These results indicate that in vivo ghrelin treatment did not inhibit the induction of $MOG_{35-55}$-reactive T cells.

### *Ghrelin does not affect induction of pathogenic autoimmune T cells*

To further confirm that $MOG_{35-55}$-reactive T cells are normally induced in ghrelin-treated mice, we evaluated if the ability of the $MOG_{35-55}$-sensitized lymphoid cells, obtained from $MOG_{35-55}$-immunized mice, to transfer EAE into naive mice could be affected by in vivo ghrelin treatment. To this aim, we immunized donor mice with $MOG_{35-55}$ and treated them every day with ghrelin or saline from immunization up to day 10. Next day, we pooled lymphocytes from spleen and LN and cultured them in the presence of $MOG_{35-55}$. Three days later, $CD4^+$ T cells were purified and injected into recipient mice as described in *Materials and Methods*. It was theoretically possible that in vivo ghrelin treatment does not

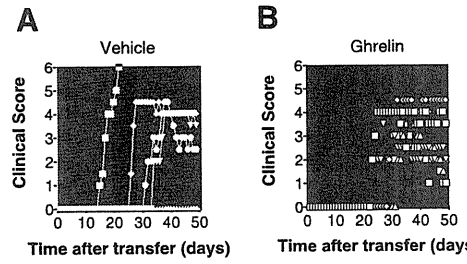


**FIGURE 4.** Effects of ghrelin treatment on the induction of encephalitogenic T cells. $MOG_{35-55}$-sensitized lymphoid cells were derived from $MOG_{35-55}$-immunized and (*A*) saline- or (*B*) ghrelin-treated mice ($n$ = 15/group). The cells were stimulated with $MOG_{35-55}$ and $CD4^+$ T cells were separated 3 days later for passive transfer of EAE into naive mice ($n$ = 5/group). Data represent individual EAE score for each mouse.

inhibit induction of $MOG_{35-55}$-reactive T cells, but would prohibit the ability to cause EAE in vivo. In postulating that this could happen, $CD4^+$ T cells from ghrelin-treated donors should be less encephalitogenic than those from saline-treated mice. The results showed that transfer of activated $CD4^+$ T cells either derived from saline- or ghrelin-treated donors induced passive EAE in the recipients, showing approximately the same clinical course and severity (Fig. 4). Thus, it can be concluded that ghrelin treatment does not affect the induction of encephalitogenic $MOG_{35-55}$-reactive $CD4^+$ T cells.

*Ghrelin decreases mRNA levels of proinflammatory cytokines in the CNS*

After demonstrating that ghrelin does not suppress the infiltration of inflammatory cells in the spinal cord, we wondered whether the cytokine milieu in the ghrelin-treated mice could be significantly altered. To answer the question, we analyzed the mRNA levels of pro- and antiinflammatory cytokines (IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, IL-6, IL-4, IL-10, and TGF-$\beta$) in the spinal cord, spleen, LN, and thymus of ghrelin- and saline-treated mice at the peak of disease (day 17) by using quantitative PCR. Although ghrelin treatment had no effect on the mRNA levels of IL-4, IL-10, and IFN-$\gamma$ in the spinal cord, spleen, LN, and thymus (data not shown), we found significantly reduced levels of TNF-$\alpha$ ($p < 0.0015$), IL-1$\beta$ ($p < 0.025$), and IL-6 ($p < 0.025$) in the spinal cord of ghrelin-treated mice, compared with saline-treated ones (Fig. 5*A*). In contrast, the level of TGF-$\beta$ showed a trend for slight elevation in the spinal cord. We also found a diminished level of TNF-$\alpha$ mRNA ($p < 0.0001$) in the spleen of ghrelin-treated mice (Fig. 5*B*), whereas we saw no significant change in any of the cytokines that we measured in LN or thymus of ghrelin-treated mice (Fig. 5, *C* and *D*). Because TNF-$\alpha$, IL-1$\beta$, and IL-6 mRNAs were selectively down-regulated in the spinal cord, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. This idea was consistent with the fact that ghrelin treatment did not inhibit the induction of $MOG_{35-55}$-reactive T cells.

*Ghrelin suppresses the proinflammatory cytokine production of LPS-stimulated monocytes*

To verify the postulate that in vivo treatment with ghrelin may ameliorate EAE by targeting monocytes, we examined in vitro effects of ghrelin on the monocytic cell line RAW 264.7 that robustly produce proinflammatory cytokines when stimulated with LPS. The RAW 264.7 line cells were first exposed to various doses of ghrelin for 1 h and then stimulated with LPS. We harvested the supernatant 2 h later and measured the levels of TNF-$\alpha$ and IL-6 by ELISA. The results revealed that prior exposure to ghrelin

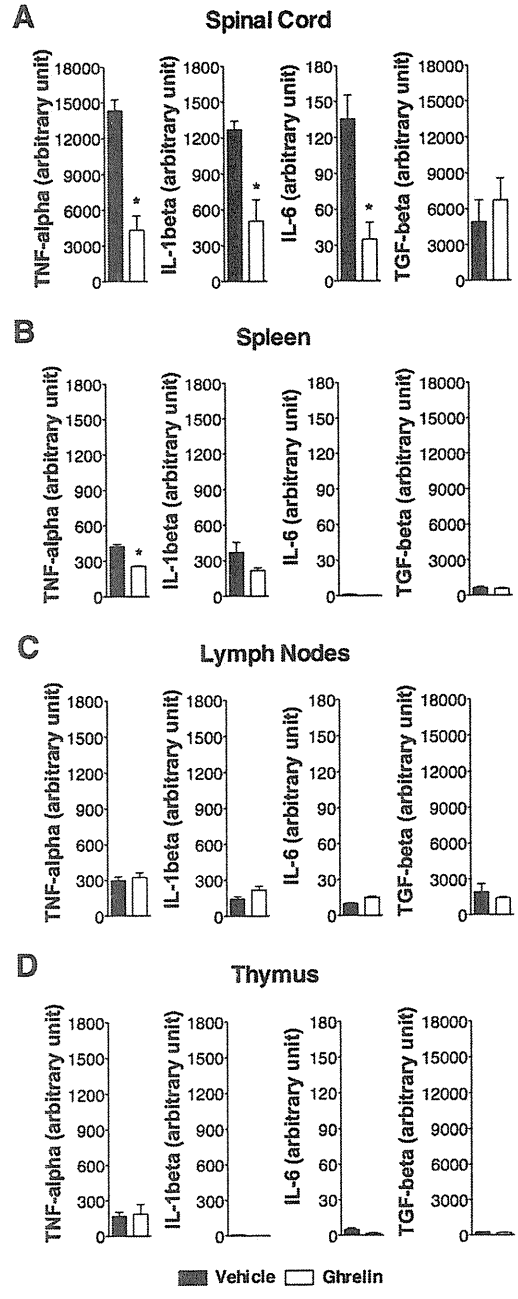


**FIGURE 5.** Proinflammatory cytokine mRNA expression during EAE in ghrelin-treated mice. Quantitative mRNA expression of proinflammatory cytokines in the spinal cord of $MOG_{35-55}$-immunized mice subjected to ghrelin or saline treatment on day 17 postimmunization ($n$ = 5/group). Total mRNA was extracted from (*A*) spinal cord, (*B*) spleen, (*C*) LN, and (*D*) thymus. The TNF-$\alpha$, IL-1$\beta$, IL-6, and TGF-$\beta$ mRNA expression was measured by real-time PCR. Data are presented as relative amount of transcript normalized to HPRT. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.025$; two-way ANOVA).

would significantly suppress the production of TNF-$\alpha$ ($p < 0.02$) and IL-6 ($p < 0.05$) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effect of ghrelin was very potent, as in addition to the effects on LPS-stimulated monocytes, even the basal production of TNF-$\alpha$ ($p < 0.008$) and IL-6 ($p < 0.03$) was significantly reduced by in vitro ghrelin treatment. Given that in vivo treatment with ghrelin could suppress the

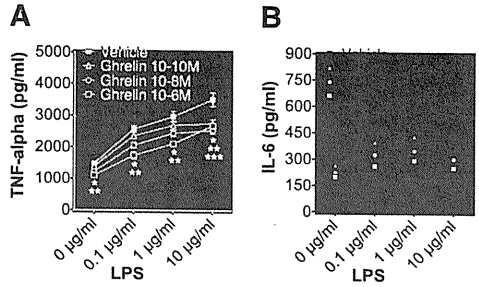


**FIGURE 6.** Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin ($10^{-6}$ M, $10^{-8}$ M, $10^{-10}$ M) 1 h before stimulation with 0.1, 1.0, and 10 $\mu$g/ml LPS. The (*A*) TNF-$\alpha$ and (*B*) IL-6 production was measured 2 h after LPS stimulation by sandwich ELISA. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. Significant differences at $10^{-6}$, $10^{-8}$, and $10^{-10}$ M ($p < 0.05$; two-way ANOVA) are depicted as *, **, and ***, respectively.

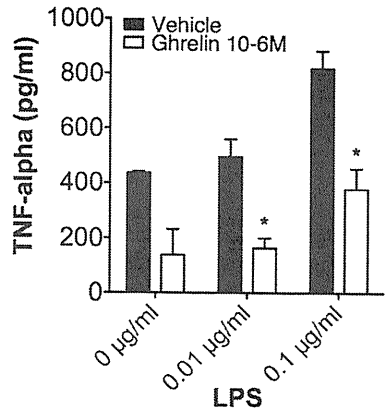


**FIGURE 8.** Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin ($10^{-6}$ M) overnight and later stimulated with 0.01 and 0.1 $\mu$g/ml LPS. Five hours after stimulation, the TNF-$\alpha$ production was measured using ELISA. Data represent mean ± SEM of duplicate samples from one out of two independent experiments. *, Significant differences between the groups ($p < 0.05$; two-way ANOVA).

development of EAE without altering histological EAE or T cell-derived cytokine balance, the ghrelin-mediated suppression of monocyte-produced TNF-$\alpha$ and IL-6 would strongly support the postulate that monocytes are the main target cells in ghrelin-mediated suppression of EAE.

*Ghrelin inhibits the expression of proinflammatory cytokines in microglia*

The proinflammatory cytokines are known to be produced not only by CNS-infiltrating macrophages but also by T cells and microglia in the course of EAE. To investigate which cells are important in the ghrelin-mediated suppression of EAE, we first examined the expression of proinflammatory cytokines in macrophages. Unexpectedly, the mRNA of IL-1$\beta$, IL-6, and TNF-$\alpha$ did not alter in CNS-infiltrating macrophages of ghrelin-treated mice compared with the control mice (Fig. 7*A*). We next examined the expression of these cytokines in other cell types also known as a source of inflammatory cytokines and found reduced expression of these cytokines in microglia (Fig. 7*B*). Additionally, the expression of inflammatory cytokines was decreased in CNS-infiltrating T cells (Fig. 7*C*). Hence, these results suggest that microglia might play a crucial role in ghrelin-mediated inhibition of EAE.

*Ghrelin inhibits the proinflammatory cytokine production of LPS-stimulated microglia*

We next examined the effect of ghrelin on microglia. To test whether ghrelin directly affects microglia, we isolated mononuclear cells from the brains of untreated mice. In untreated non-EAE mice, most (~77%) of the brain mononuclear cells were $CD11b^+$ cells, and the majority of $CD11b^+$ cells (~95%) were considered as $CD45^{low}$ microglia cells. Among these mononuclear cells, $CD19^+$ B cells were <0.1% and $CD3^+CD45^+$ T cells were 1–1.5%. We cultured the isolated mononuclear cells in the presence of ghrelin overnight and stimulated them with LPS in different doses for 5 h. The TNF-$\alpha$ levels in the culture supernatant were measured by using ELISA. In the presence of ghrelin, the TNF-$\alpha$ levels were significantly reduced (Fig. 8). These results suggest that ghrelin directly affects microglia by reducing the production of inflammatory cytokines.

## Discussion

Starvation is known to have immunosuppressive effects (24–26). Although little was known about the mechanistic link between starvation and immunity, recent studies have shed light on the immunomodulatory potency of a range of feeding regulatory hormones such as leptin and NPY. For example, serum leptin is decreased after acute starvation in parallel with immunosuppression or Th2 bias, whereas exogenous leptin would correct the altered Th1/Th2 balance toward Th1 (27, 28). In contrast, NPY is increased after starvation. Exogenous NPY would shift the Th1/Th2 balance toward Th2 and can ameliorate the severity of EAE (29). Interestingly, both peptide hormones are linked to ghrelin in an endocrine feedback system (30). Ghrelin itself is increased after

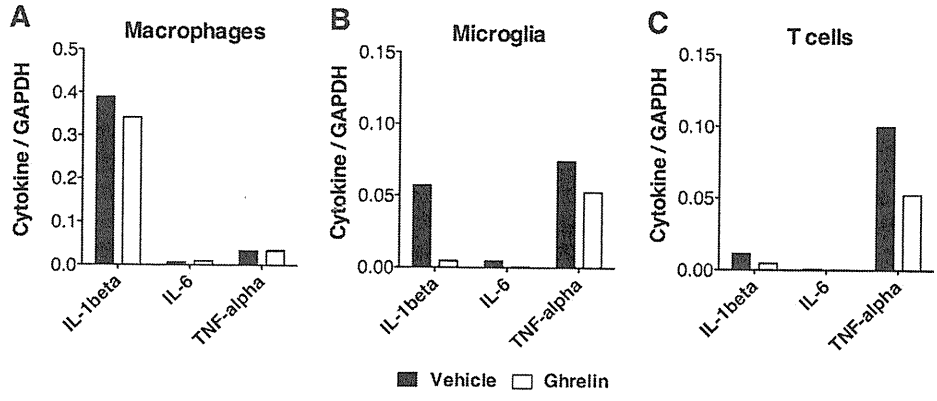


**FIGURE 7.** Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (*A*) macrophages, (*B*) microglia, and (*C*) T cells obtained on day 20 postimmunization from the spinal cords of $MOG_{35-55}$-immunized mice treated with ghrelin or saline. The IL-1$\beta$, IL-6, and TNF-$\alpha$ mRNA expression levels were measured by real-time PCR. Data are presented as relative amount of transcript normalized to the housekeeping gene GAPDH.

starvation, and it can potently stimulate the release of NPY in the CNS (12). Moreover, ghrelin shows antagonistic effects against leptin (31). Although the available data on the action of ghrelin on leptin or NPY may not be extrapolated to speculate about its role in the immune system, we decided to explore whether ghrelin may exhibit beneficial effects in the modulation of EAE. Furthermore, ghrelin was reported to have protective effects on endotoxic shock in rats (32). Additionally, the wide range of GHS-R expression within the immune cells strongly suggested the immunomodulatory potential of ghrelin (6). Considering its endocrine interactions, ghrelin becomes an interesting candidate for the in vivo modulation of EAE.

To evaluate the effects of ghrelin on the immune system in vivo, we used the representative EAE model induced with $MOG_{35-55}$ in B6 mice. Subcutaneous injections of ghrelin significantly suppressed EAE severity, especially after the peak of disease, while the EAE onset occurred almost similarly in both ghrelin- and sham-treated mice. Priming phase treatment (days 1–10) as well as effector phase treatment (days 11–20) also showed disease-suppressing effects, suggesting a modulatory role of ghrelin during all phases of disease. The unacylated ghrelin form, des-acyl ghrelin, failed to suppress EAE, demonstrating that the disease suppression was mediated by the GHS-R.

The histological findings at day 17 were similar in all animals regardless of the applied treatment. The inflammatory cell infiltration and demyelination occurred in both groups, suggesting a ghrelin effect independent of cell trafficking at the peak of disease. Moreover, we found by FACS analysis that the number of mononuclear cells isolated from the spinal cord and their composition did not significantly alter among ghrelin- and sham-treated mice at the same time point. Our data showed no statistically significant changes in the examined cell subsets, which supported the histological findings of unaffected immune cell traffic to the CNS. This discrepancy between analogous inflammatory status in the spinal cord on the one hand and less severe disease on the other hand in ghrelin-treated mice was remarkable, suggesting cytokine regulation as the possible mechanism of EAE suppression.

Leptin and NPY both influence the Th1/Th2 balance in opposing directions (27–29). Since ghrelin is the most potent NPY-releasing hormone and NPY suppresses EAE by a Th2 bias (29), we examined whether ghrelin affects the Th1/Th2 balance similar to NPY and if its potential mechanism of EAE suppression is primarily mediated on immune cells or secondarily through NPY release. To investigate the effect of ghrelin on the cytokine balance, we measured the cytokine responses of $MOG_{35-55}$-primed T cells from mice treated with ghrelin or saline. The evaluated IFN-$\gamma$, IL-17, and IL-4 levels as well as the proliferative response did not significantly alter between ghrelin- and sham-treated mice. Underlying these observations, we conclude that the suppression of EAE mediated by ghrelin does not affect the T cell-derived cytokine balance. To further address whether ghrelin acts via the NPY pathway, we determined the encephalitogenic potential of $CD4^+$ T cells from ghrelin-treated mice to cause passive EAE in syngeneic recipients. We treated donor animals with ghrelin or saline for 10 days after priming with $MOG_{35-55}$, and lymphoid cells from the mice were stimulated with $MOG_{35-55}$. Three days later, $CD4^+$ T cell blasts were isolated and transferred to naive mice. The $CD4^+$ T cells from ghrelin-treated mice did not differ from those from saline-treated mice in the ability to mediate passive EAE, indicating that ghrelin does not primarily affect induction of encephalitogenic $CD4^+$ T cells in vivo. While NPY attenuates EAE by a Th2 bias of encephalitogenic $CD4^+$ T cells (29), our findings likely suggest that ghrelin interacts independently of NPY in the amelioration of EAE.

To further clarify the mechanism of ghrelin-mediated EAE suppression, we examined the mRNA levels of several cytokines of ghrelin- and sham-treated mice at the peak of disease. Our data demonstrate significantly reduced levels of the proinflammatory cytokines TNF-$\alpha$, IL-1$\beta$, and IL-6 in the spinal cord and lower levels of TNF-$\alpha$ in the spleen of ghrelin-treated mice. In contrast, the level of TGF-$\beta$ showed a trend for slight elevation in the spinal cord. The importance of TNF-$\alpha$ for initiating and sustaining inflammation is well described, as well as its essential role in the development of acute EAE (33, 34). The proinflammatory role of IL-1$\beta$ and IL-6 in the immunopathology of EAE is also generally accepted (35–38). Thus, the inhibition of TNF-$\alpha$, IL-1$\beta$, and IL-6 must be considered as an important mechanism in the ghrelin-mediated EAE suppression.

Given the selective down-modulation of the proinflammatory cytokines, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. However, the analysis of infiltrating cells and residential microglia revealed that the suppression of proinflammatory cytokines was prominently led by microglia. A decreased expression of these cytokines was also observed in infiltrating T cells. Considering that the transfer of T cells obtained from ghrelin-treated mice induced a similar disease course compared with control mice, the reduction of proinflammatory cytokines in microglia might be important in the ghrelin-mediated suppression of EAE.

In conclusion, the present study demonstrates for the first time to our knowledge that the gastric hormone ghrelin suppresses actively induced EAE by inhibiting production of the proinflammatory cytokines TNF-$\alpha$, IL-1$\beta$, and IL-6 with microglia as the main target cells. These findings support an antiinflammatory property of ghrelin, shedding light on its role in immune-endocrine interactions. Consequently, we speculate that ghrelin may serve as an antiinflammatory drug to control human CNS pathology involving the production of proinflammatory cytokines.

## Disclosures

The authors have no financial conflicts of interest.

## References


1. Deghenghi, R., M. M. Cananzi, A. Torsello, C. Battisti, E. E. Muller, and V. Locatelli. 1994. GH-releasing activity of Hexarelin, a new growth hormone releasing peptide, in infant and adult rats. *Life Sci.* 54: 1321–1328.
2. Howard, A. D., S. D. Feighner, D. F. Cully, J. P. Arena, P. A. Liberator, C. I. Rosenblum, M. Hamelin, D. L. Hreniuk, O. C. Palyha, J. Anderson, et al. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273: 974–977.
3. Smith, R. G., K. Cheng, W. R. Schoen, S. S. Pong, G. Hickey, T. Jacks, B. Butler, W. W. Chan, L. Y. Chaung, F. Judith, et al. 1993. A nonpeptidyl growth hormone secretagogue. *Science* 260: 1640–1643.
4. Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660.
5. Date, Y., M. Kojima, H. Hosoda, A. Sawaguchi, M. S. Mondal, T. Suganuma, S. Matsukura, K. Kangawa, and M. Nakazato. 2000. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 141: 4255–4261.
6. Hattori, N., T. Saito, T. Yagyu, B. H. Jiang, K. Kitagawa, and C. Inagaki. 2001. GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. *J. Clin. Endocrinol. Metab.* 86: 4284–4291.
7. Hosoda, H., M. Kojima, H. Matsuo, and K. Kangawa. 2000. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem. Biophys. Res. Commun.* 279: 909–913.
8. Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. *Nature* 409: 194–198.
9. Tschop, M., D. L. Smiley, and M. L. Heiman. 2000. Ghrelin induces adiposity in rodents. *Nature* 407: 908–913.
10. Muccioli, G., M. Tschop, M. Papotti, R. Deghenghi, M. Heiman, and E. Ghigo. 2002. Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. *Eur. J. Pharmacol.* 440: 235–254.

11. Nagaya, N., T. Itoh, S. Murakami, H. Oya, M. Uematsu, K. Miyatake, and K. Kangawa. 2005. Treatment of cachexia with ghrelin in patients with COPD. *Chest* 128: 1187–1193.
12. Cowley, M. A., R. G. Smith, S. Diano, M. Tschop, N. Pronchuk, K. L. Grove, C. J. Strasburger, M. Bidlingmaier, M. Esterman, M. L. Heiman, et al. 2003. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37: 649–661.
13. Shintani, M., Y. Ogawa, K. Ebihara, M. Aizawa-Abe, F. Miyanaga, K. Takaya, T. Hayashi, G. Inoue, K. Hosoda, M. Kojima, et al. 2001. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 50: 227–232.
14. Dixit, V. D., E. M. Schaffer, R. S. Pyle, G. D. Collins, S. K. Sakthivel, R. Palaniappan, J. W. Lillard, Jr., and D. D. Taub. 2004. Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *J. Clin. Invest.* 114: 57–66.
15. Wassem, T., M. Duxbury, H. Ito, S. W. Ashley, and M. K. Robinson. 2008. Exogeneous ghrelin modulates release of pro- and anti-inflammatory cytokines in LPS-stimulated macrophages through distinct signaling pathways. *Surgery* 143: 334–342.
16. Chorny, A., P. Anderson, E. Gonzalez-Rey, and M. Delgado. 2008. Ghrelin protects against experimental sepsis by inhibiting high-mobility group box 1 release and by killing bacteria. *J. Immunol.* 180: 8369–8377.
17. Gonzalez-rey, E., A. Chorny, and M. Delgado. 2006. Therapeutic action of ghrelin in a mouse model of colitis. *Gastroenterology* 130: 1707–1720.
18. Granado, M., T., Priego, A. I., Martin, A., Villanua, and A. Lopez-Caldron. 2005. Anti-inflammatory effect of the ghrelin agonist growth hormone-releasing peptide-2 (GHRP-2) in arthritic rats. *Am. J. Physiol.* 288: E486–E492.
19. Li, W. G., D. Gavrila, X. Liu, L. Wang, S. Gunnlaugsson, L. L. Stoll, M. L. McCormick, C. D. Sigmund, C. Tang, and N. L. Weintraub. 2004. Ghrelin inhibits proinflammatory responses and nuclear factor-κB activation in human endothelial cells. *Circulation* 109: 2221–2226.
20. Wu, R., W. Dong, X. Cui, M. Zhou, H. H. Simms, T. S. Ravikumar, and P. Wang. 2007. Ghrelin down-regulates proinflammatory cytokines in sepsis through activation of the vagus nerve. *Ann. Surg.* 245: 480–486.
21. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor Vβ expression of encephalitogenic T cells. *Eur. J. Immunol.* 25: 1951–1959.
22. Zhang, B., T. Yamamura, T. Kondo, M. Fujiwara, and T. Tabira. 1997. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J. Exp. Med.* 186: 1677–1687.
23. Miyamoto, K., S. Miyake, M. Mizuno, N. Oka, S. Kusunoki, and T. Yamamura. 2006. Selective COX-2 inhibitor celecoxib prevents experimental autoimmune encephalomyelitis through COX-2-independent pathway. *Brain* 129: 1984–1992.
24. Chan, J. L., G. Matarese, G. K. Shetty, P. Raciti, I. Kelesidis, D. Aufiero, V. De Rosa, F. Perna, S. Fontana, and C. S. Mantzoros. 2006. Differential regulation of metabolic, neuroendocrine, and immune function by leptin in humans. *Proc. Natl. Acad. Sci. USA* 103: 8481–8486.
25. Kuchroo, V. K., and L. B. Nicholson. 2003. Immunology: fast and feel good? *Nature* 422: 27–28.
26. Wing, E. J., D. M. Magee, and L. K. Barczynski. 1988. Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity. *Immunology* 63: 677–682.
27. Lord, G. M., G. Matarese, J. K. Howard, R. J. Baker, S. R. Bloom, and R. I. Lechler. 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394: 897–901.
28. Sanna, V., A. Di Giacomo, A. La Cava, R. I. Lechler, S. Fontana, S. Zappacosta, and G. Matarese. 2003. Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. *J. Clin. Invest.* 111: 241–250.
29. Bedoui, S., S. Miyake, Y. Lin, K. Miyamoto, S. Oki, N. Kawamura, A. Beck-Sickinger, S. von Horsten, and T. Yamamura. 2003. Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY1 receptor-specific inhibition of autoreactive Th1 responses in vivo. *J. Immunol.* 171: 3451–3458.
30. Kalra, S. P., and P. S. Kalra. 2003. Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. *Endocrine* 22: 49–56.
31. Kalra, S. P., N. Ueno, and P. S. Kalra. 2005. Stimulation of appetite by ghrelin is regulated by leptin restraint: peripheral and central sites of action. *J. Nutr.* 135: 1331–1335.
32. Chang, L., J. Zhao, J. Yang, Z. Zhang, J. Du, and C. Tang. 2003. Therapeutic effects of ghrelin on endotoxic shock in rats. *Eur. J. Pharmacol.* 473: 171–176.
33. Glabinski, A. R., B. Bielecki, J. A. Kawczak, V. K. Tuohy, K. Selmaj, and R. M. Ransohoff. 2004. Treatment with soluble tumor necrosis factor receptor (sTNFR):Fc/p80 fusion protein ameliorates relapsing-remitting experimental autoimmune encephalomyelitis and decreases chemokine expression. *Autoimmunity* 37: 465–471.
34. Xanthoulea, S., M. Pasparakis, S. Kousteni, C. Brakebusch, D. Wallach, J. Bauer, H. Lassmann, and G. Kollias. 2004. Tumor necrosis factor (TNF) receptor shedding controls thresholds of innate immune activation that balance opposing TNF functions in infectious and inflammatory diseases. *J. Exp. Med.* 200: 367–376.
35. Furlan, R., A. Bergami, E. Brambilla, E. Butti, M. G. De Simoni, M. Campagnoli, P. Marconi, G. Comi, and G. Martino. 2007. HSV-1-mediated IL-1 receptor antagonist gene therapy ameliorates $MOG_{35-55}$-induced experimental autoimmune encephalomyelitis in C57BL/6 mice. *Gene Ther.* 14: 93–98.
36. Okuda, Y., S. Sakoda, H. Fujimura, Y. Saeki, T. Kishimoto, and T. Yanagihara. 1999. IL-6 plays a crucial role in the induction phase of myelin oligodendrocyte glucoprotein 35–55 induced experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 101: 188–196.
37. Okuda, Y., S. Sakoda, Y. Saeki, T. Kishimoto, and T. Yanagihara. 2000. Enhancement of Th2 response in IL-6-deficient mice immunized with myelin oligodendrocyte glycoprotein. *J. Neuroimmunol.* 105: 120–123.
38. Sutton, C., C. Brereton, B. Keogh, K. H. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203: 1685–1691.

**The Memorial Thesis of the Best Poster Award (Recommended article)**
**Recommender: Chairman of The 36th Annual Meeting of The Japan Society for Clinical Immunology, Prof. Nobuyuki MIYASAKA**

# Retinoid signals and Th17-mediated pathology

Christian KLEMANN, Benjamin JE RAVENEY, Shinji OKI and Takashi YAMAMURA

*Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan*

(Received January 21, 2009)

**summary**

For many years, CD4+ effector T cells were categorized into two subsets: T helper type 1 (Th1) and type 2 (Th2) cells. More recent research has refined this model, delineating further subsets; in particular, Th17 cells, activated CD4+ T cells characterised by the production of the cytokine IL-17. Autoantigen-specific Th17 cells are associated with pathology in a number of animal models of organ-specific autoimmune disease and evidence is mounting that Th17 cells are also critical in human autoimmunity.

Retinoids, a family of compounds that bind to and activate retinoic acid receptors (RARs and RXRs), are able to alter CD4+ T cell differentiation *in vitro* though agonism and antagonism of a range of retinoid receptors. For example, *all-trans* retinoic acid (ATRA) inhibits Th17 differentiation and instead promotes the upregulation of Foxp3, a key transcription factor in regulatory T cells. Importantly, treatment with retinoids can modulate Th17-mediated autoimmunity: experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis (MS), is ameliorated by ATRA administration due to suppression of both the differentiation and the function of Th17 cells. In this review, we discuss the unveiled molecular mechanism and the possible clinical application of retinoids for the treatment of human Th17-mediated autoimmune diseases.

**Key words**——Retinoids; AM80; ATRA; EAE; Th17; IL-17; RORγt; Treg; Foxp3; IL-10

## Introduction

During an immune response, CD4+ T cells can become activated in an antigen-specific manner and direct the nature of the response by activating and counter-regulating other leukocyte populations. Upon activation, naive CD4+ T cells can differentiate into a range of cell types, which elaborate a tailored response depending on the nature of the immune insult. These differentiated cell types can be effector CD4+ T cells, including Th1, Th2, and Th17 types, or CD4+ regulatory T cells (Treg) that deviate the function of other immune cells, including Tr3, Th10, iTreg types. Many factors in the microenvironment during CD4+ T cell activation tune these differentiation processes, including signals from the antigen-presenting cell and the cytokine milieu. In addition, retinoids can act to drive the generation of Treg and inhibit the differentiation of pro-inflammatory Th17 cells. In this review, we will discuss the potential of retinoids to influence Treg and Th17 responses, in order to treat autoimmune diseases.

国立精神・神経センター神経研究所免疫研究部

## Discovery of Th17 cells

The dichotomous classification of effector CD4+ T cells based on their function, into Th1 and Th2 cells was first reported in 1986[1]. It was demonstrated that naïve Th cells differentiate to two functional classes of cell during an immune response, Th1 cells, which produce interferon (IFN)-γ and are involved in cell-mediated immunity and organ-specific autoimmunity, and Th2 cells, which secrete interleukin (IL)-4 and are involved in extracellular immunity and pathogenesis of asthma and allergy. Furthermore, a key finding was that these Th subsets were able to negatively regulate each other, explaining how a single Th-type response is established following a particular immune insult. This pioneering work fuelled the understanding of the immune system for many years and remained largely unchallenged.

Multiple sclerosis (MS), the human autoimmune inflammatory disease of the central nervous system (CNS), is characterised by perivascular infiltrates in the brain that display hallmarks of delayed-type hypersensitivity (DTH). This DTH response was ascribed to Th1 cells[2] and, following this research, MS and other autoimmune diseases were thus thought to be mediated by Th1 cells[3]. Experimental autoim-

mune encephalomyelitis (EAE), a well-established model of cell-mediated autoimmunity[4,5], is also thought to be generated by the action of Th1 cells, which was supported by the fact that autoreactive Th1 cells are able to transfer the disease. Furthermore, IL-12p40-deficient mice, which are unable to mount Th1 responses, were resistant to induction of EAE, supporting the hypothesis that the disease is a Th1-mediated disorder. Thus, it was predicted that the administratration of IFN-$\gamma$, the key effector cytokine produced by Th1 cells, should generate more severe EAE and conversely the inhibition of the effect of IFN-$\gamma$ should reduce EAE. In fact, the opposite is the case: IFN-$\gamma$ administration ameliorates disease, whilst neutralization of IFN-$\gamma$ with blocking antibodies leads to worsen clinical outcome[6,7]. Furthermore, mice deficient in either IFN-$\gamma$, or IFN-$\gamma$ receptor, which lack Th1 responses are also susceptible to more severe EAE[8]. Similar observations have also been made in other models of autoimmunity such as adjuvant-induced arthritis[9].

Despite such contradicting data, the Th1/Th2 paradigm was upheld for more than two decades until new data regarding the role of IL-23 allowed the formulation of a improved hypothesis of Th differentiation. IL-12 and IL-23 are both heterodimeric cytokines consisting of a shared p40 subunit, but unlike in IL-12 where this molecule combines with a p35 subunit, IL-23 possesses a unique p19 subunit[10]. The related structure of these cytokines was able to solve a long-standing puzzle: why mice deficient for p40, part of both IL-12 and IL-23, were protected from EAE induction, whilst IL-12p35 deficient mice develop worse disease. Thus, when IL-23 p19 deficient mice were generated, and were found to be protected from EAE induction, it was concluded that the protective effect in p40 mice was unrelated to IL-12[11]. Furthermore, anti-IL-23 treatment also leads to protection from EAE[12]. Despite this similar structural make up of these two cytokines, their biological activities differ greatly: IL-12 controls the differentiation of Th1 cells, whilst IL-23 does not. Instead, IL-23 was found to be associated with effector T cells that produced large amounts of IL-17A, IL-17F, IL-21, and IL-22[10,13]. The pivotal roles of IL-23-associate T cells were unveiled by a series of experiments that showed that the adoptive transfer of these T cells caused severe EAE[14,15], neutralization of IL-17 via mAb treatment ameliorated EAE, and IL-17-deficient mice developed less severe EAE with a delayed onset[16,17]. Other autoimmune diseases have also been shown to be Th17-mediated, such as rheumatoid arthritis[18], and in animal models of this disease severity is reduced in IL-17-deficient mice and by blockade of IL-17 signalling[19,20].

Earlier work demonstrated that addition of IL-23 to $CD4^+$ T cell cultures led to IL-17 production by T cells that were initially referred to as Th-IL-17 cells[21], but later this became abbreviated to Th17 cells[22]. At first, IL-23 was assumed to be a factor important for the *de novo* generation of Th17 cells. It soon became clear that this was not the case, since naive T cells do not express the IL-23 receptor[13,23]. Instead, a combination of the immune suppressive cytokine TGF-$\beta$ and pro-inflammatory IL-6 has been identified as the differentiating factors of Th17 cells in mice[13,23,24]. Additionally, there may be a requirement for IL-1$\beta$ in the differentiation of human Th17 cells. Interestingly, the differentiation of naïve $CD4^+$ T cells activated in the presence of TGF-$\beta$ alone induces the generation of induced Tregs, which express the transcription factor Foxp3[25]. At this point of time, IL-23 is believed to play a crucial role in the expansion and maintenance of Th17 cells.

Th17 cells are characterized by the expression of the transcription factor retinoid acid-related orphan nuclear receptor-$\gamma$t (ROR$\gamma$t)[26], ROR$\alpha$[27], and signal transducer and activator of transcription-3 (STAT3)[28,29]. When EAE is induced in ROR$\gamma$t-deficient mice, disease has a delayed onset and is of a milder form than in wildtype mice[26].

In humans, there is a growing body of evidence that implicates Th17 cells in autoimmune processes. There are increased levels of transcripts for IL-17 and IL-6 in the CNS lesions of patients with MS[30], and in such lesions, as well as in cerebrospinal fluid from MS patients, IL-17-secreting lymphocytes have been detected[31,32]. Th17 responses have also been associated with the human autoimmune disorders such as psoriasis[33,34], rheumatoid arthritis[35], and Crohn's disease and ulcerative colitis[36].

### Vitamin A and its metabolites

Vitamin A (retinyl ester) plays essential roles in a number of physiological functions throughout the body, including vision, embryonic development, bone and blood metabolism, gene transcription, and immune functions[37~39]. The recommended daily allowances of vitamin A range from 300 $\mu$g/day in children to 1200 $\mu$g/day for lactating women[40]. It is important to note that vitamin A uptake deficiency compromises normal immune responses. Retinol, the

most common metabolite of dietary vitamin A, can be either absorbed by the gut following ingestion, or generated from provitamins such as betacarotene[37~39]. Retinol is then processed into retinal and retinoic acid (RA)[41]. All-trans-retinoic acid (ATRA), 9-cis RA, and 11-cis retinal are the most active metabolites found in the body[38,42]. 11-cis retinal is required for the synthesis of rhodopsin and thus is essential for vision. ATRA and 9-cis RA bind to retinoic acid receptors (RARs) and retinoid X receptors (RXRs) and via these interactions control transcription of a variety of genes, both activation and repression[37~39,43]. ATRA preferably binds to the RARs, whilst 9-cis RA can bind to both receptor classes[37,38]. As RARs and RXRs both have three isotypes, $\alpha$, $\beta$, and $\gamma$, which can combine to a form many different heterodimers and homodimers, the range of retionid affinity for this range of receptors can lead to a great number of possible effects.

**Natural and synthetic retinoids in the clinic[44]**

The term retinoids is applied to a family of compounds that bind to and activate retinoic acid receptors (RARs and RXRs), resulting in a range of possible biological responses. Some natural retinoids, such as ATRA (Tretinoin), 9-cis RA (Alitretinoin), and 13-cis RA (Isotretinoin), are currently already used in the clinic. As the clinical use of natural retinoids is limited by their pharmacological profile, including instability, poor bioavailability, and possible side effect due to the nonspecific receptor binding of those natural retinoids, a number synthetic retinoids have been generated. These include mono-aromatic synthetic retinoids (second generation), such as Acitretin and Etretinate, and poly-aromatic synthetic retinoids (third generation), such as Tamibarotene (AM80), Tazarotene, and Targretin (LGD1069) (See Table 1). The aromatic rings found in the second and third generation retinoids confer a higher stability and resistance to heat/oxidation, increased half-lives, a higher potency, and improved spectrum of action with receptor specificities.

The most common clinical use of retinoids to date is in the treatment of acute promyelocytic leukemia (APL) and Kaposi's sarcoma[45,46]. APL results from an abnormal fusion protein PML-RAR$\alpha$, formed due to a t (15; 17) (q22; q12) chromosomal translocation, inducing abnormal promyelocytic cell proliferation. The efficacy of retinoid treatment of APL has been reported to be due to the promotion of granulocytic differentiation and maturation. Importantly, the synthetic retinoids exhibit greater potency in APL treatment compared with their natural occurring counterparts: for example the third generation retinoid Tamibarotene (AM80) is effective in ATRA-unresponsive APL patients[47]. Further studies have also suggested that autoimmune disease are effectively targeted with these new generation of retinoids, such of the skin disease psoriasis[48].

Retinoids have been studied for over 20 years as potential therapeutic agents in a variety of autoimmune models, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, type I diabetes, and lupus[49~53]. However, many clinical trials examining the potential for retionids in treating such diseases have indicated a efficacy and intolerable side effects[54]. Previously, as ATRA was shown to effect T cell differentiation, both suppressing Th1 development and enhancing Th2 development[55], the amelioration of autoimmune disease by retinoid treatment was attributed to a deviation of immune from Th1 to Th2. More recently, with an enhanced understanding of Th differentiation outcomes, the effect of retinoid treatment on Th17 and Treg cell development and function has reawakened interested in use of retinoids to treat immune disorders. Furthermore, this research

Table 1. Retinoids in clinical use

| Name | Receptors | Clinical use |
|---|---|---|
| ATRA (Tretinoin) | pan-RAR | APL, Acne |
| 9-cis RA (Alitretinoin) | pan-RAR, pan-RXR | Kaposi sarcoma |
| 13-cis RA (Isotretinoin) | — | Acne |
| Etretinate | — | Psoriasis |
| Acitretin | pan-RAR | Psoriasis |
| Tamibarotene (AM80) | RAR$\alpha/\beta \gg \gamma$ | APL |
| Tazarotene | RAR$\beta/\gamma \gg \alpha$ | Acne |
| Targretin (LGD1069) | pan-RXR | Cutaneous T lymphoma |

has been facilitated with the availability of a new range of enhanced, synthetic, receptor-specific retinoid compounds.

### Retinoids and Th17 cells

It is now well established that Th17 cells constitute a distinct subset of inflammatory T cells, which are characterized by the production of IL-17 and the expression of the transcription factors RORγt and RORα[56]. Th17 differentiation is enhanced in CD4+ T cells that are induced to overexpress RORγt or RORα and overexpression of both these genes has an even greater effect[27]. Interference with either of these genes reduces the propensity of CD4+ T cells to differentiate into Th17 cells and if both RORγt and RORα are knocked out, Th17 differentiation is prevented[27]. Retinoids strongly suppress the *in vitro* production of IL-17 by polyclonal TCR stimulation of naïve CD4+ T cells in the presence of IL-6 and TGF-β[57~59]. This inhibition of Th17 cell function is accompanied by downregulation of RORγt and upregulation of Foxp3[57~59]. The suppressive effect of retinoids on Th17 cell differentiation has been shown to be mediated via RARα[57,59]. Therefore, it is highly conceivable that retinoids, particularly those with high affinity for RARα, may be of use in the clinic to treat Th17-mediated pathology. However, the ability of retinoids to inhibit Th17 differenition may be of limited use in alleviating in human autoimmune disease, as when patients present, T cell activation and differentiation is likely to be at an advance stage. It is therefore essential to consider the effect of retinoid on terminally differentiated T cells.

We have observed that the restimulation of *in vitro* differentiated Th17 cells and activated CD4+ splenocytes from mice with active Th17-mediated autoimmune disease results in the production of high-levels of IL-17 secretion, which is reduced by the addition of the synthetic retinoid AM80. Additionally, AM80 treatment of these cells also downregulates RORγt expression. We demonstrated inhibition of Th17 cell function at AM80 doses as low as 0.1-10 nM, however maximal suppression was achieved with concentrations in the order of 10-100 nM (unpublished observation). It is important to note that higher doses of retinoid treatment have been shown to have a wide-ranging anti-proliferative and immunosuppressive effect on T cells (unpublished data and[60]). Therefore, for translation to the clinic at the applicable human dose of retinoid must be determined to ensure efficacy of retinoid treatment without undesirable pan-immunosuppression. Xiao *et al.* demonstrated the potential application of ATRA to the treatment of EAE, however, in this report ATRA administration also generated significant anti-proliferative effects[61]. We have determined that the therapeutic effect of the synthetic retinoid AM80 on EAE occurs at much lower doses than ATRA. This is achievable as the pharmacological profile of AM80 allows its administration via the oral route, which may also be desirable in treatment of human disease. Critically, we were able to demonstrate that although these doses strongly downregulated Th17 mediated pathology, no general immunosuppression was observed.

An interesting observation is that retinoid treatment may actually already be in current use for Th17-mediated diseases. For some time, retinoids have been utilised as a standard treatment for psoriasis[62]. More recently, psoriasis has been linked with Th17 responses[33,34], thus it is likely that retinoids can be applied to treat a wider range of immune pathologies, in particular those associated with Th17 dysfunction.

### Retinoids and Foxp3+ regulatory T cells

The majority of Treg cells, CD4+ T cells that are able to counter-suppress populations of T cells, express the transcription factor Foxp3. The function of these cells is critical in the maintenance of self tolerance and defects in Foxp3 lead to widespread autoimmunity. This is observed in both mice (Scurfy mice) and men, (e.g. X-linked (IPEX) syndrome)[63~65]. Foxp3+ CD4+ Treg can be generated in the thymus (natural Treg) or be generated in the periphery following activation (induced Treg). *In vitro*, naïve T cells can also be induce to differentiate into Foxp3+ Treg by activation in a particular cytokine environment. One such stimulus for Treg differentiation is TGF-β, a cytokine that is also required for Th17 differentiation. Interestingly, it has been reported that retinoids, including ATRA and AM80, are also able to alter T cell differentiation, inducing Foxp3+ CD4+ T cells ([57-59,66-69] and unpublished data). Such differentiation occurs in the presence of TGF-β1 despite the addition of IL-6, conditions that would normally promote Th17 differentiation. It has been suggested that retinoids mediate this effect by disrupting the signalling of IL-6 and IL-23 through receptor downregulation and by Smad3-dependent amplification of the TGF-β signalling[61].

Intriguingly, a subset of CD103+ dendritic cells found in the lamina propria, and mesenteric lymph

nodes have the ability to supply ATRA to T cells during antigen-priming[69,70], conversely splenic dendritic cells do not produce significant amounts of ATRA[67~69]. The upregualtion of Foxp3 induced by ATRA is dependent on both TGF-$\beta$ and retinoid signalling. Neutralizing antibody against TGF-$\beta$ blocks the induction of Foxp3$^+$ CD4$^+$ T cells[69] and inhibition of the enzyme required for ATRA synthesis or blocking retinoid receptor signalling also prevents Foxp3 induction[58,68,69].

Despite the upregulation of Foxp3 generated by retinoids *in vitro*, treatment of EAE with retinoids suppresses Th17 cell function without promoting Foxp3 expression[58,61]. It has been suggested that a lack of TGF-$\beta$ may be the limiting factor[58] or that the adjuvant-induced pro-inflammatory cytokine milieu, including IL-1, TNF-$\alpha$ and IL-6, may actively suppress Treg conversion[61]. Nevertheless, retinoid treatment is effective in suppressing the *de novo* differentiation of Th17 cells *in vivo* as well as *in vitro*. Therefore, we suggest re-evaluation of previous findings showing the beneficial effects of retinoid treatment in autoimmune diseases.

## Conclusion

We have summarized the recent findings that Th17 cells are a major component in the pathology of many autoimmune diseases and that retinoids, especially synthetic one such as AM80 with a higher stability, increased half-life, a higher potency, and improved spectrum of actions with receptor specificities, are potent candidates for disease intervention. Recent study has pointed out the existence of Foxp3/RORγt double positive T cell subset, which produces IL-10 instead of IL-17[71]. In addition, treatment of activated T cells with TGF-$\beta$ and IL-6 in the absence of terminal differentiation by IL-23 rendered them to produce IL-10, which are protective for EAE when transferred adoptively[72]. IL-10 has been shown to be another important regulatory component for autoimmune responses. Interestingly, our recent work suggested that long-term treatment with retinoids such as AM80 might cause downregulation of IL-10 production. Therefore, retinoids have multifunctional target on immune systems (downregulation of IL-6/IL-23 signals, Th17 function, and IL-10 production and upregulation of Foxp3, Treg function and TGF-$\beta$ signals) and thus, clinical application of retinoids should be determined by the evaluation of each component to achieve the maximum effect of retinoids.

## Acknowledgements

The authors would like to thank all the contributors to this work.

## References

1) Mosmann TR, Coffman RL : TH1 and TH2 cells : different patterns of lymphokine secretion lead to different functional properties, *Annu Rev Immunol* **7** : 145-173, 1989.
2) Cher DJ, Mosmann TR : Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones, *J Immunol.* **138** : 3688-3694, 1987.
3) Lassmann H, Ransohoff RM : The CD4-Th1 model for multiple sclerosis : a critical [correction of crucial] re-appraisal, *Trends Immunol.* **25** : 132-137, 2004.
4) Gold R, Linington C, Lassmann H : Understanding pathogenesis and therapy of multiple sclerosis via animal models : 70 years of merits and culprits in experimental autoimmune encephalomyelitis research, *Brain* **129** : 1953-1971, 2006.
5) Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB : T cell response in experimental autoimmune encephalomyelitis (EAE) : role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire, *Annu Rev Immunol* **20** : 101-123, 2002.
6) Billiau A, Heremans H, Vandekerckhove F, Dijkmans R, Sobis H, Meulepas E, Carton H : Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma, *J Immunol.* **140** : 1506-1510, 1988.
7) Voorthuis JA, Uitdehaag BM, De Groot CJ, Goede PH, van der Meide PH, Dijkstra CD : Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats, *Clin Exp Immunol.* **81** : 183-188, 1990.
8) Ferber IA, Brocke S, Taylor-Edwards C, Ridgway W, Dinisco C, Steinman L, Dalton D, Fathman CG : Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE), *J Immunol* **156** : 5-7, 1996.
9) Jacob CO, Holoshitz J, Van der Meide P, Strober S, McDevitt HO : Heterogeneous

effects of IFN-gamma in adjuvant arthritis, *J Immunol.* **142** : 1500-1505, 1989.
10) McGeachy MJ, Cua DJ : The link between IL-23 and Th17 cell-mediated immune pathologies, *Semin Immunol* **19** : 372-376, 2007.
11) Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD : Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain, *Nature* **421** : 744-748, 2003.
12) Chen Y, Langrish CL, McKenzie B, Joyce-Shaikh B, Stumhofer JS, McClanahan T, Blumenschein W, Churakovsa T, Low J, Presta L, Hunter CA, Kastelein RA, Cua DJ : Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis, *J Clin Invest* **116** : 1317-1326, 2006.
13) Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK : Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, *Nature* **441** : 235-238, 2006.
14) Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, Toyka KV, Gold R : Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis, *Cell Immunol* **237** : 123-130, 2005.
15) Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ : IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, *J Exp Med* **201** : 233-240, 2005.
16) Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y : IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis, *J Immunol* **177** : 566-573, 2006.
17) Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C : A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, *Nat Immunol* **6** : 1133-1141, 2005.
18) Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, Tanaka S, Kodama T, Akira S, Iwakura Y, Cua DJ, Takayanagi H : Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction, *J Exp Med* **203** : 2673-2682, 2006.
19) Bush KA, Farmer KM, Walker JS, Kirkham BW : Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein, *Arthritis Rheum* **46** : 802-805, 2002.
20) Nakae S, Nambu A, Sudo K, Iwakura Y : Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice, *J Immunol* **171** : 6173-6177, 2003.
21) Bettelli E, Kuchroo VK : IL-12- and IL-23-induced T helper cell subsets : birds of the same feather flock together, *J Exp Med.* **201** : 169-171, 2005.
22) Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL : Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17, *J Biol Chem* **278** : 1910-1914, 2003.
23) Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B : TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, *Immunity* **24** : 179-189, 2006.
24) Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT : Transforming growth factor-beta induces development of the T(H)17 lineage, *Nature* **441** : 231-234, 2006.
25) Ziegler SF : FOXP3 : of mice and men, *Annu Rev Immunol.* **24** : 209-226, 2006.
26) Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR : The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells, *Cell* **126** : 1121-1133, 2006.
27) Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C : T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma, *Immunity* **28** : 29-39, 2008.
28) Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea J J : In-

terleukin-2 signaling via STAT5 constrains T helper 17 cell generation, *Immunity* **26** : 371-381, 2007.

29) Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, Dong C : STAT3 regulates cytokine-mediated generation of inflammatory helper T cells, *J Biol Chem* **282** : 9358-9363, 2007.

30) Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, Langer-Gould A, Strober S, Cannella B, Allard J, Klonowski P, Austin A, Lad N, Kaminski N, Galli SJ, Oksenberg JR, Raine CS, Heller R, Steinman L : Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis, *Nat Med* **8** : 500-508, 2002.

31) Matusevicius D, Kivisakk P, He B, Kostulas N, Ozenci V, Fredrikson S, Link H : Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis, *Mult Scler* **5** : 101-104, 1999.

32) Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L : Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis, *Am J Pathol* **172** : 146-155, 2008.

33) Teunissen MB, Koomen CW, de Waal Malefyt R, Wierenga EA, Bos JD : Interleukin-17 and interferon-gamma synergize in the enhancement of proinflammatory cytokine production by human keratinocytes, *J Invest Dermatol* **111** : 645-649, 1998.

34) van Beelen AJ, Teunissen MB, Kapsenberg ML, de Jong EC : Interleukin-17 in inflammatory skin disorders, *Curr Opin Allergy Clin Immunol* **7** : 374-381, 2007.

35) Aarvak T, Chabaud M, Miossec P, Natvig JB : IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells, *J Immunol* **162** : 1246-1251, 1999.

36) Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, Kistner EO, Schumm LP, Lee AT, Gregersen PK, Barmada MM, Rotter JI, Nicolae DL, Cho JH : A genome-wide association study identifies IL23R as an inflammatory bowel disease gene, *Science* **314** : 1461-1463, 2006.

37) Chambon P : A decade of molecular biology of retinoic acid receptors, *Faseb J* **10** : 940-954, 1996.

38) Mark M, Ghyselinck NB, Chambon P : Function of retinoid nuclear receptors : lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis, *Annu Rev Pharmacol Toxicol* **46** : 451-480, 2006.

39) Stephensen CB : Vitamin A, infection, and immune function, *Annu Rev Nutr* **21** : 167-192, 2001.

40) USDA : Composition of Foods Raw, Processed, Prepared USDA National Nutrient Database for Standard Reference, 2008.

41) Napoli JL : Retinoic acid : its biosynthesis and metabolism, *Prog Nucleic Acid Res Mol Biol* **63** : 139-188, 1999.

42) Duester G : Families of retinoid dehydrogenases regulating vitamin A function : production of visual pigment and retinoic acid, *Eur J Biochem* **267** : 4315-4324, 2000.

43) Bourguet W, Germain P, Gronemeyer H : Nuclear receptor ligand-binding domains : three-dimensional structures, molecular interactions and pharmacological implications, *Trends Pharmacol Sci* **21** : 381-388, 2000.

44) Fukasawa H, Kagechika H, Shudo K : [Retinoid therapy for autoimmune diseases], *Nihon Rinsho Meneki Gakkai Kaishi.* **29** : 114-126, 2006.

45) Fenaux P, Chomienne C, Degos L : Treatment of acute promyelocytic leukaemia, *Best Pract Res Clin Haematol* **14** : 153-174, 2001.

46) Krown SE : Therapy of AIDS-associated Kaposi's sarcoma : targeting pathogenetic mechanisms, *Hematol Oncol Clin North Am* **17** : 763-783, 2003.

47) Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A, Karasuno T, Takeuchi M, Miyawaki S, Ueda R, Naoe T, Ohno R : Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid, *Blood.* **90** : 967-973, 1997.

48) Kuenzli S, Saurat JH : Retinoids for the treatment of psoriasis : outlook for the future, *Curr Opin Investig Drugs* **2** : 625-630, 2001.

49) Massacesi L, Abbamondi AL, Giorgi C, Sarlo F, Lolli F, Amaducci L : Suppression of experimental allergic encephalomyelitis by retinoic acid, *J Neurol Sci* **80** : 55-64, 1987.

50) Nozaki Y, Yamagata T, Sugiyama M, Ikoma S, Kinoshita K, Funauchi M : Anti-inflammatory effect of all-trans-retinoic acid in inflammatory arthritis, *Clin Immunol* **119** : 272-279, 2006.
51) Osanai M, Nishikiori N, Murata M, Chiba H, Kojima T, Sawada N : Cellular retinoic acid bioavailability determines epithelial integrity : Role of retinoic acid receptor alpha agonists in colitis, *Mol Pharmacol* **71** : 250-258, 2007.
52) Perez de Lema G, Lucio-Cazana FJ, Molina A, Luckow B, Schmid H, de Wit C, Moreno-Manzano V, Banas B, Mampaso F, Schlondorff D : Retinoic acid treatment protects MRL/lpr lupus mice from the development of glomerular disease, *Kidney Int* **66** : 1018-1028, 2004.
53) Zunino SJ, Storms DH, Stephensen CB : Diets rich in polyphenols and vitamin A inhibit the development of type I autoimmune diabetes in nonobese diabetic mice, *J Nutr* **137** : 1216-1221, 2007.
54) Beehler BC, Brinckerhoff CE, Ostrowski J : Selective retinoic acid receptor ligands for rheumatoid arthritis, *Curr Opin Investig Drugs* **5** : 1153-1157, 2004.
55) Iwata M, Eshima Y, Kagechika H : Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors, *Int Immunol* **15** : 1017-1025, 2003.
56) Steinman L : A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage, *Nat Med* **13** : 139-145, 2007.
57) Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, O´Shea JJ : Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway, *Blood* **111** : 1013-1020, 2008.
58) Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H : Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid, *Science* **317** : 256-260, 2007.
59) Schambach F, Schupp M, Lazar MA, Reiner SL : Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation, *Eur J Immunol* **37** : 2396-2399, 2007.
60) Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, McFarlin DE, Scott DE : Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course, *J Immunol* **154** : 450-458, 1995.
61) Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK : Retinoic acid increases Foxp3$^+$ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression, *J Immunol* **181** : 2277-2284, 2008.
62) Thaci D : Long-term data in the treatment of psoriasis, *Br J Dermatol* **159** Suppl 2 : 18-24, 2008.
63) Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD : The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3, *Nat Genet* **27** : 20-21, 2001.
64) Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F : Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse, *Nat Genet* **27** : 68-73, 2001.
65) Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME : X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy, *Nat Genet* **27** : 18-20, 2001.
66) Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ : All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation, *J Exp Med* **204** : 1765-1774, 2007.
67) Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F : A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism, *J Exp Med* **204** : 1757-1764, 2007.
68) Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH : Vitamin A metabolites induce gut-homing FoxP3$^+$ regulatory T cells, *J Immunol* **179** : 3724-3733, 2007.

69) Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y : Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid, *J Exp Med* **204** : 1775–1785, 2007.

70) Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY : Retinoic acid imprints gut-homing specificity on T cells, *Immunity* **21** : 527–538, 2004.

71) Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, Riethmacher D, Si-Tahar M, Di Santo JP, Eberl G : In vivo equilibrium of proinflammatory IL-17$^+$ and regulatory IL-10$^+$ Foxp3$^+$ RORgamma t$^+$ T cells, *J Exp Med* **205** : 1381–1393, 2008.

72) McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ : TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T (H)-17 cell-mediated pathology, *Nat Immunol* **8** : 1390–1397, 2007.

*Neuropathology and Applied Neurobiology* (2009), **35**, 16–35 doi: 10.1111/j.1365-2990.2008.00947.x

# Protein microarray analysis identifies human cellular prion protein interactors

J. Satoh*†, S. Obayashi*, T. Misawa*, K. Sumiyoshi*, K. Oosumi* and H. Tabunoki*

**Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, and †Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan*

J. Satoh, S. Obayashi, T. Misawa, K. Sumiyoshi, K. Oosumi and H. Tabunoki (2009) *Neuropathology and Applied Neurobiology* **35**, 16–35

**Protein microarray analysis identifies human cellular prion protein interactors**

**Aims:** To obtain an insight into the function of cellular prion protein (PrPC), we studied PrPC-interacting proteins (PrPIPs) by analysing a protein microarray. **Methods:** We identified 47 novel PrPIPs by probing an array of 5000 human proteins with recombinant human PrPC spanning amino acid residues 23–231 named PR209. **Results:** The great majority of 47 PrPIPs were annotated as proteins involved in the recognition of nucleic acids. Coimmunoprecipitation and cell imaging in a transient expression system validated the interaction of PR209 with neuronal PrPIPs, such as FAM64A, HOXA1, PLK3 and MPG. However, the interaction did not generate proteinase K-resistant proteins. KeyMolnet, a bioinformatics tool for analysing molecular interaction on the curated knowledge database, revealed that the complex molecular network of PrPC and PrPIPs has a significant relationship with AKT, JNK and MAPK signalling pathways. **Conclusions:** Protein microarray is a useful tool for systematic screening and comprehensive profiling of the human PrPC interactome. Because the network of PrPC and interactors involves signalling pathways essential for regulation of cell survival, differentiation, proliferation and apoptosis, these observations suggest a logical hypothesis that dysregulation of the PrPC interactome might induce extensive neurodegeneration in prion diseases.

Keywords: cellular prion protein, KeyMolnet, protein microarray, protein–protein interaction

Published online *Article Accepted* on 23th February 2008

## Introduction

Prion diseases are a group of neurodegenerative disorders affecting both animals and humans [1,2]. The great majority of prion diseases are transmissible, and characterized by intracerebral accumulation of an abnormal prion protein (PrPSc) that is identical in amino acid sequence to the cellular isoform (PrPC) encoded by the *PRNP* gene. PrPC is expressed widely in neural and non-neural tissues at the highest level in neurones in the central nervous system (CNS) [3]. PrPSc differs biochemically from PrPC by its β sheet-enriched structure, detergent insolubility, limited proteolysis by proteinase K, a slower turnover rate and infectivity. Previous studies suggested that the protein conformational conversion of α-helix-rich PrPC into β sheet-rich PrPSc involves a homotypic interaction between endogenous PrPC and incoming or *de novo* generated PrPSc via a post-translational process mediated by as yet unidentified species-specific auxiliary factor(s) named 'protein X' [4,5].

At present, the biological function of PrPC remains largely unknown. Several lines of PrPC-deficient mice were established independently by different gene-targeting strategies [6–8]. All of them exhibited normal early development and complete protection against scrapie infection. These observations indicate that PrPC is dispensable for embryonic development, but is pivotal for inducing prion

Correspondence: Jun-ichi Satoh, Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204–8588, Japan. Tel: +81 42 4958678; Fax: +81 42 4958678; E-mail: satoj@my-pharm.ac.jp

diseases. Several *in vitro* studies suggested a role of PrPC in neuritogenesis [9,10], neuronal cell adhesion [11] and a receptor for neurotrophic factors [12]. More consistently, many studies indicated that an octapeptide repeat region of PrPC with a copper-binding capacity exhibits an antioxidant activity [13]. However, none of previous findings provided an adequate explanation for mild phenotypes of PrPC-deficient mice.

A number of previous studies, by employing mainly the yeast two-hybrid (Y2H) screening system, identified a wide variety of PrPC-interacting proteins (PrPIPs). They include synapsin I [14], glial fibrillary acidic protein [15], amyloid precursor-like protein 1 [16], heat shock protein Hsp60 [17–19], the Hsp cofactor STI-1 [20], the antiapoptotic molecule Bcl-2 [21], signal-transducing adapters such as Grb2 [14], ZAP70 [22] and 14-3-3 [23], neurotrophin receptor interacting MAGE homolog [24], tubulin [25], heterogeneous ribonuclear protein A2/B1 [26], casein kinase 2 [27], plasminogen [28], laminin receptor precursor [29], laminin [9] and vitronection [30]. Most of these molecules play a key role in signal-transducing events essential for neuronal function. However, none of them could serve as the chaperone 'protein X'.

The Y2H system is a powerful approach to identify novel protein–protein interactions. However, Y2H screening requires a lot of time and effort, and is often criticized for detecting the interactions unrelated to the physiological setting, and obtaining high rates of false positive interactors caused by spontaneous activation of reporter genes and self-activating bait proteins [31,32]. Recently, protein microarray technology has been established for rapid, systematic and less expensive screening of thousands of protein–protein interactions in a high-throughput fashion [33,34]. The array includes numerous protein targets of various functional classes immobilized on a single glass slide. The protein microarray has important applications in the areas not only of basic biological research on a whole-proteome scale, but also of drug discovery research of target identification [35,36].

In order to establish a therapeutic intervention targeted on prion propagation, it is essential to clarify the biological function of PrPC and the pathological implication of PrPSc, and equally important to identify all human PrPIPs, some of which potentially serve as a candidate for 'protein X'. The present study was designed to identify a comprehensive profile of the human PrPC interactome by analysing a high-density protein microarray, and to obtain an insight into the PrPC–PrPIPs network.

## Materials and methods

### Preparation of a V5-tagged PrP probe for microarray analysis

Human embryonic kidney cells HEK293, whose genome was modified for the Flp-In system (Flp-In 293; Invitrogen, Carlsbad, CA), contain a single Flp recombination target (FRT) site targeted for the site-specific recombination, integrated in a transcriptionally active locus of the genome, where it stably expresses the *lacZ*–Zeocin fusion gene driven from the pFRT/*lacZ*eo plasmid under the control of SV40 early promoter. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (feeding medium) with inclusion of 100 μg/ml zeocin, as described previously [37].

To prepare the probe for protein microarray analysis, the gene encoding a truncated form of human PrPC spanning amino acid residues 23–231 named PR209 was amplified by polymerase chain reaction (PCR) using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and the primer sets listed in Table S1 online. The PCR product was then cloned into a mammalian expression vector pSecTag/FRT/V5-His TOPO (Invitrogen) to produce a fusion protein with a C-terminal V5 tag, a C-terminal polyhistidine (6xHis) tag and an N-terminal Ig κ-chain secretion signal. This vector, together with the Flp recombinase expression vector pOG44 (Invitrogen), was transfected in Flp-In 293 cells by Lipofectamine 2000 reagent (Invitrogen). A stable cell line was established after incubating the transfected cells for 1 month in the feeding medium with inclusion of 100 μg/ml hygromycin B. In this system, the recombinant protein was secreted into the culture medium after the Ig κ-chain secretion signal sequence was processed by an endogenous signal peptidase-mediated cleavage.

To purify the V5-tagged PR209 protein, the serum-free culture supernatant was harvested, and concentrated at a 1/40 volume by centrifugation on an Amicon Ultra-15 filter (Millipore, Bedford, MA). It was then purified by the HIS-select spin column (Sigma, St. Louis, MO), and concentrated at a 1/10 volume by centrifugation on a Centricon-10 filter (Millipore). The protein concentration was determined by a Bradford assay kit (Bio-Rad, Hercules, CA). The purity and specificity of the probe were verified by Western blot analysis using mouse monoclonal anti-V5 antibody (Invitrogen), mouse monoclonal anti-

PrP antibody 3F4 (Dako, Tokyo, Japan) and rabbit polyclonal antibody C20 specific for the sequence close to the C-terminus of PrPC (Santa Cruz Biotechnology, Santa Cruz, CA). To determine the status of glycosylation, 5 μg of the probe protein was deglycosylated by incubating it at 37°C for 1.5 h with 5000 U peptide N-glycosidase F (New England BioLabs, Beverly, MA), followed by separation on the gel [37].

## Protein microarray analysis

The present study utilized the ProtoArray human protein microarray v3.0 (Invitrogen). It contains approximately 5000 recombinant GST-tagged human proteins expressed by the baculovirus expression system and purified under native conditions by using gluthathione affinity chromatography to ensure the preservation of native structure, post-translational modifications and proper functionality of target proteins [36,38]. They were spotted in duplicate on a nitrocellulose-coated glass slide. The target proteins cover a wide range of biologically important proteins selected from the human ultimate open reading frame (ORF) clone collection (Invitrogen). The probe is spatially accessible to all parts of target proteins on the array, which protrude from the glass slide surface via the N-terminal GST fusion tag serving as a spacer. The complete list is shown in Table S2 online. The proteins are spotted in an arrangement of 4 × 12 subarrays equally spaced in vertical and horizontal directions. Each subarray includes 20 × 20 spots, composed of 76 positive and negative control spots (C), 222 human target proteins (H), and 102 blanks and empty spots (B) (Figure 1**b**). The 14 positive control spots include four of an Alexa Fluor 647-labelled antibody (row 1, columns 1, 2; row 14, columns 13, 14), six of a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 14, columns 15–20), and four of a concentration gradient of V5 protein (row 15, columns 5–8). The 62 negative control spots include six of a concentration gradient of bovine serum albumin (BSA) (row 1, columns 3–8), four of a concentration gradient of a rabbit anti-GST antibody (row 1, columns 9–12), four of a concentration gradient of calmodulin (row 1, columns 13–16), 16 of a concentration gradient of GST (row 1, columns 17–20; row 2, columns 1–12), 10 of buffer only (row 15, columns 1, 2, 9–16), eight of human IgG subclasses (row 15, columns 17–20; row 16, columns 1–4), 12 of Invitrogen internal controls (row 4, columns 9, 10; row 6, columns 15–18; row 7, columns 9, 10, 15, 16; row 8, columns 13, 14), and two of an antibiotin antibody (row 15, columns 3, 4).

Non-specific binding was blocked by incubating the array for 90 min in the PBST blocking buffer composed of 1% BSA and 0.1% Tween 20 in phosphate-buffered saline (PBS). Then, it was incubated for 30 min at 4°C with the probe described above at a concentration of 200 μg/ml in the probing buffer composed of 1% BSA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05% Triton X-100 and 5% glycerol in PBS. The array was washed three times with the probing buffer, and then incubated for 30 min at 4°C with mouse monoclonal anti-V5 antibody labelled with Alexa Fluor 647 (Invitrogen) at a concentration of 260 ng/ml in the probing buffer. Then, the array was washed three times with the probing buffer, and scanned by the GenePix 4200 A scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm. The data were analysed by using the ProtoArray Prospector software v3.0 (Invitrogen), following acquisition of the microarray lot-specific information, which compensates inter-lot variations in protein concentrations identified by the post-printing quality control. According to the manufacturer-recommended setting of the ProtoArray Prospector software, the spots showing the background-subtracted signal intensity value greater than the median plus three standard deviations of all the fluorescence intensities were considered as having significant interactions. The Z-score, an indicator for statistical significance of binding specificity, was calculated as the background-subtracted signal intensity value of the target protein minus the average of the background-subtracted signal intensity value from the negative control distribution, divided by the standard deviation of the negative control distribution.

## Bioinformatics analysis

The gene expression pattern of mouse orthologues of PrPIPs in the brain was searched on the Allen Brain Atlas database [39], an anatomically comprehensive digital atlas containing the expression patterns of more than 20 000 genes in the adult mouse brain analysed by high-throughput *in situ* hybridization methods (http://www.brain-map.org).

The interaction of PrPC with PrPIPs was searched on the Biomolecular Interaction Network database (BIND) (http://bond.unleashedinformatics.com). Functional

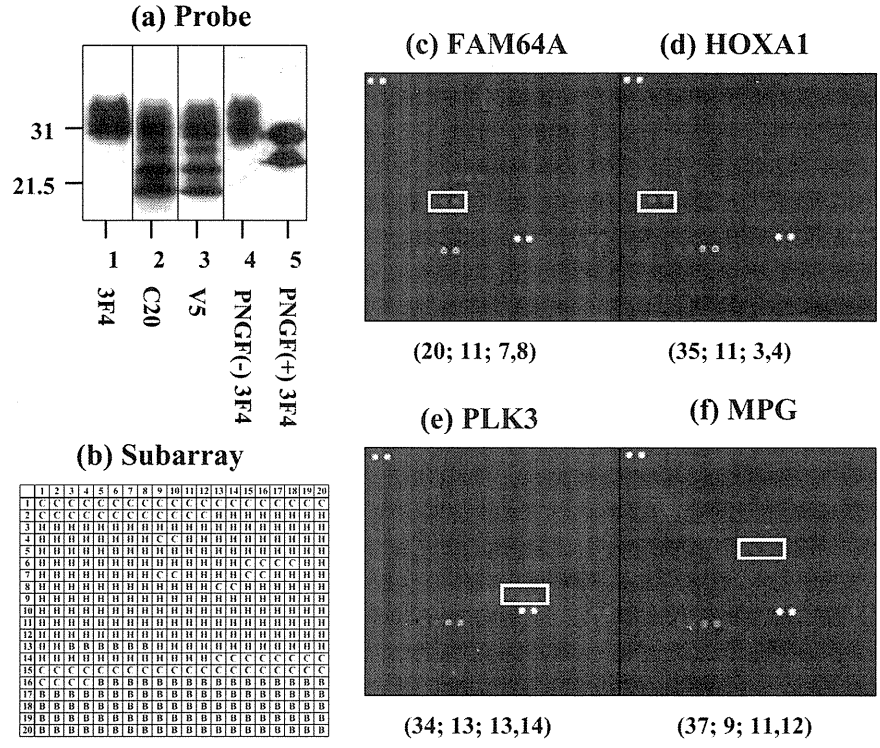


**Figure 1.** Protein microarray analysis. (a) Western blot of PR209 probe. The lanes (1–5) represent the immunolabelling with the antibodies following: (1) 3F4, (2) C20, (3) V5, (4) 3F4 before treatment with peptide N-glycosidase F (PNGase F) and (5) 3F4 after treatment with PNGase F. (b) The layout of subarray. The high-density protein microarray (5000 proteins, duplicate spots each) utilized in the present study contains 4 × 12 subarrays. Each subarray includes 20 × 20 spots. They are composed of 76 control spots (C) including 14 positive and 62 negative control spots, 222 human target proteins (H) and 102 blanks and empty spots (B), as described in *Materials and methods*. (c) FAM64A. The spot location is subarray 20, row 11, columns 7, 8. (d) HOXA1. Subarray 35, row 11, columns 3, 4. (e) PLK3. Subarray 34, row 13, columns 13, 14. (f) MPG. Subarray 37, row 9, columns 11, 12. PR209 interactors located on different subarrays (c–f) are indicated by an enclosed yellow line. It is worthy to note that in each subarray, positive control spots composed of an Alexa Fluor 647-labelled antibody (row 1, columns 1, 2; row 14, columns 13, 14), a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 14, columns 15–20; signals visible on the higher concentration), and a concentration gradient of V5 protein (row 15, columns 5–8; signals visible on the higher concentration) are identified as positive, whereas negative control spots composed of a concentration gradient of BSA (row 1, columns 3–8), a concentration gradient of a rabbit anti-GST antibody (row 1, columns 9–12), a concentration gradient of calmodulin (row 1, columns 13–16), a concentration gradient of GST (row 1, columns 17–20; row 2, columns 1–12), buffer only (row 15; columns 1, 2, 9–16), human IgG subclasses (row 15, columns 17–20; row 16, columns 1–4), Invitrogen internal controls (row 4, columns 9, 10; row 6, columns 15–18; row 7, columns 9, 10, 15, 16; row 8, columns 13, 14), and an antibiotin antibody (row 15, columns 3, 4) are found as negative.

annotation of PrPIPs was searched by the web-accessible program named Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2007, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) (http://david.abcc.ncifcrf.gov) [40]. It covers more than 40 annotation categories, including Gene Ontology terms, protein–protein interactions, protein functional domains, disease associations, biological pathways, sequence general features, homologies, gene functional summaries and tissue expressions. By importing the list of Entrez gene IDs of PrPIPs, this program creates the functional annotation chart, an annotation term-focused view that lists annotation terms and their associated genes under study. To avoid excessive counting of duplicated genes, the Fisher's exact statistics is calculated based on corresponding DAVID gene IDs by which all redundancies in original IDs are removed.

The molecular network of PrPIPs was analysed by the software named KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan) [41]. It operates on a comprehensive knowledge database, composed of information on relationships among human genes, molecules, diseases, pathways and drugs, carefully curated by expert biologists from review articles, literature and public

databases. They are categorized into the core contents collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed database and Human Reference Protein database.

By importing the list of Entrez gene IDs, KeyMolnet automatically provides corresponding molecules as a node on networks [41,42]. Among various network-searching algorithms, the 'N-points to N-points' search extracts the molecular network with the shortest route connecting the starting-point molecules and the end-point molecules. The generated network was compared side by side with 346 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations (approximately 90 000 sets) and X = the sigma variable that defines incidental agreements.

$$\text{score} = -\log_2\left(\sum_{x=O}^{Min(C,V)} f(x)\right)$$

$$f(x) = {}_{C}C_{X} \cdot {}_{T\text{-}C}C_{V\text{-}x} / {}_{T}C_{V}$$

## Immunoprecipitation and Western blot analysis

PR209, the N-terminal half of PR209 (amino acid residues 23–121), the C-terminal half of PR209 (amino acid residues 122–231), and the ORF of family with sequence similarity 64, member A (FAM64A), polo-like kinase 3 (PLK3), N-methylpurine-DNA glycosylase (MPG) and homeobox A1 (HOXA1) were amplified by PCR using PfuTurbo DNA polymerase and the primer sets listed in Table S1 online. They were then cloned into the mammalian expression vector p3XFLAG-CMV7.1 (Sigma) or pCMV-Myc (Clontech, Mountain View, CA) to express a fusion protein with an N-terminal Flag or Myc tag. At 48 h after co-transfection of the vectors, HEK293 cells were homogenized in M-PER lysis buffer (Pierce, Rockford, IL) supplemented with a cocktail of protease inhibitors (Sigma). In limited experiments, a proteasome inhibitor MG-132 (Merck-Calbiochem, Tokyo, Japan) was added at a final concentration of 10 μM in the culture medium during the last 24 h before harvest. After preclearance, the supernatant was incubated at 4°C for 3 h with mouse monoclonal anti-Flag M2 affinity gel (Sigma), rabbit polyclonal anti-Myc-conjugated agarose (Sigma) or the same amount of normal mouse or rabbit IgG-conjugated agarose (Santa Cruz Biotechnology). After several washes, the immunoprecipitates were processed for Western blot analysis using rabbit polyclonal anti-Myc antibody (Sigma) and mouse monoclonal anti-FLAG M2 antibody (Sigma). The specific reaction was visualized using a chemiluminescence substrate (Pierce).

To determine the proteinase K-resistant property of PR209, the cells were homogenized in M-PER lysis buffer without inclusion of protease inhibitors. The protein extract was then incubated at 37°C for 30 min with 5 μg/ml recombinant proteinase K (Roche Diagnostics, Mannheim, Germany), followed by adding phenylmethylsulphonyl fluoride at a final concentration of 5 mM, according to the methods described previously [43]. Proteins were precipitated by adding 6% trichloroacetic acid. After centrifugation at 4°C for 15 min at 16 100 g, the pellets were washed with cold acetone, and processed for Western blot analysis using 3F4 antibody.

To determine the detergent-insoluble property of PR209, the cells were homogenized in a lysis buffer containing 100 mM NaCl, 10 mM EDTA, 10 mM Tis (pH 7.4), 0.5% Nonidet P-40 and 0.5% sodium deoxycholate, according to the methods described previously [44]. The lysate was centrifuged at 4°C for 10 min at 2000 g to remove debris. Then, the supernatant was further centrifuged at 4°C for 1 h at 16 100 g to separate detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions. They were processed for Western blot analysis using 3F4 antibody. HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

## Cell imaging analysis

PR209 and the ORF of FAM64A, PLK3, MPG and HOXA1 were amplified by PCR using PfuTurbo DNA polymerase and the primer sets listed in Table S1 online. They were then cloned into the mammalian expression vector pDsRed-Express-C1 (Clontech), pEYFP-C1 (Clontech), pcDNA3.1/NT/GFP-TOPO (Invitrogen) or pcDNA3.1/CT/GFP-TOPO (Invitrogen) to express a fusion protein with an N-terminal or C-terminal DsRed, EYFP or GFP tag. At 24–48 h after co-transfection of the vectors, the cells were