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Prostaglandin- E_2 Regulation of Tumor Necrosis Factor Receptor Release in Human Monocytic THP-1 Cells

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Recent *in vitro* studies indicate that tumor necrosis factor (TNF) production in human monocytic THP-1 cells is suppressed by action of arachidonic acid metabolite prostaglandin- E_2 (PGE₂). PGE₂ stimulation of human monocytic cell line THP-1 demonstrates that PGE₂ not only regulates TNF activity at production levels, but does so through the release of two soluble TNF receptors (BP-55, BP-75) as well. PGE₂ can thus exert a regulatory effect on TNF biologic activity by interfering with its ability to reach cell membrane receptors. THP-1 cells were activated with PGE₂ for either 2- or 6-hr time periods, and the supernatants subsequently tested by ELISA to quantitate the levels of soluble receptor released. In addition, we examined mechanisms of receptor shedding by investigating the rate of membrane internalization and the role of serine proteases. PGE₂-stimulated THP-1 cells showed soluble 55- and 75-kDa TNF receptor release levels which exceeded that of spontaneous release at both 2- and 6-hr activation periods. The numbers of both membrane TNF receptors were significantly upregulated as well in PGE₂-activated cells, whereas the levels of 55- and 75-kDa TNF receptor mRNA levels remained unchanged. Thus, PGE₂ induces TNF receptor release primarily at posttranscriptional levels. Inhibition of serine proteases with Pefabloc, a phenylmethylsulfonyl fluoride analog, resulted in the inhibition of both spontaneous and PGE₂-stimulated release. Treatment of THP-1 cells with *N*-ethylmaleimide and low-temperature incubation, both known to block membrane internalization, also blocked spontaneous and PGE₂-induced release. Internalization and cleavage by protease are therefore critical factors in PGE₂-induced release of soluble TNF receptor shedding. © 1996 Academic Press, Inc.

INTRODUCTION

Tumor necrosis factor (TNF)² is a polypeptide secreted by activated macrophages and other immune

effector cells in response to inflammation, immunologic reactions, and physiological stress in acute tissue injury or sepsis. TNF is cytotoxic for tumor cells and appears to be involved in the pathogenesis of endotoxic shock as well (1). Infusion of human TNF into rats elicits a condition known as cachexia, involving hypotension and hemorrhagic necrosis of various vital organs (1). TNF is also associated with activation and growth of immune cells including macrophages, B- and T-lymphocytes, endothelial cells, neutrophils, and fibroblasts (2–5).

Like many cytokines, the specific binding of TNF to its receptor molecules is the essential first step in propagating the biological activities of TNF. The TNF ligand–receptor interaction activates signal transduction pathways, leading to the expression of TNF's wide range of functions. The existence of two distinct TNF receptors (TNF-R) has been determined with molecular weights of 55 and 75 kDa (6–8).

Soluble forms of the two TNF receptors have recently been isolated in urine from normal individuals, urine from individuals with chronic inflammatory disease (9, 10), and sera from patients with various types of cancers (11, 12). The soluble forms of the 55- and 75-kDa receptors are of molecular weights 30 and 40 kDa respectively, and consist of the NH₂-terminal extracellular domains of their membrane-expressed counterparts. These soluble form receptors are cleaved and released by a variety of cells, including activated macrophages, monocytes, and tumor cells. The soluble receptors have the ability to bind to and subsequently inactivate soluble TNF both *in vivo* and *in vitro*. This binding activity of soluble cytokine receptors has suggested a general phenomenon for the regulation of TNF activity (13), yet the precise kinetics of the regulation remains unclear. The inhibition expressed through TNF ligand–receptor binding *in vivo* leads to its classification as TNF blocking protein (14).

The host antitumor response, in which TNF plays an integral role, has been found to elicit a series of reac-

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² Abbreviations used: TNF, tumor necrosis factor; TNF-R, tumor necrosis factor-receptor; PGE₂, prostaglandin E_2 ; FBS, fetal bovine

serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbant assay; LPS, lipopolysaccharide; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

tions involving the endocrine system. The theory of neuroendocrine influence over immune functions has developed from evidence of nerve endings in organs and tissues of the immune system such as the thymus gland, bone marrow, spleen, and lymph nodes (15). Recent studies have additionally documented the ability of TNF and other cytokines to stimulate the synthesis and release of certain hormones. Further investigation has revealed that not only do cells of the immune system influence those of the neuroendocrine system, but that the endocrine system, particularly the hypothalamic-pituitary axis, exerts an effect on immune cells as well. A particular hormone of interest which appears to be associated with TNF is prostaglandin-E₂ (PGE₂), an arachidonic acid metabolite and a potent immunosuppressive agent. Lipopolysaccharide (LPS) is a strong inducer of TNF on monocytes, which, in turn, stimulates the production of PGE₂. Also, PGE₂ has been found to have a regulatory effect on cytokines (16). Exogenous PGE₂ has been found to suppress LPS-induced TNF mRNA accumulation in a dose-dependent manner (17).

The focus of this study is to investigate the effects of PGE₂ on TNF receptors, based on its participation in modulation of TNF biologic activity. Regulation of TNF receptor levels may further elucidate the mechanistic details of receptor release, as well as possible parameters of neuroendocrine involvement. As macrophages play a critical role in TNF and TNF receptor production and release, *in vitro* studies were performed using human monocytic cell line THP-1. THP-1 cells produce high levels of TNF, and it closely mirrors characteristics found in normal activated macrophages.

We examined the effect of stimulating THP-1 cells with PGE₂ on TNF receptor release, as well as mRNA and surface receptor expression. PGE₂ activation of THP-1 cells resulted in the decrease of TNF release, as well as the increase of both forms of soluble TNF receptors, allowing us to postulate the existence of two distinct modes of TNF activity regulation.

MATERIALS AND METHODS

Cell Culture

THP-1 is a nonadherent, human, monocytic cell line which was obtained from the American Type Culture Collection (Rockville, MD). These cells were passed twice a week in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL). The cells were cultured in plastic flasks (Corning Glass Works, Corning, NY) and incubated in a 37°C, CO₂ incubator.

Activation of THP-1 Cells by PGE₂

THP-1 cells were seeded in 24-well tissue culture plates (Corning Glass Works) at a concentration of 1

× 10⁶ cells per 1 ml, per well. Cells were activated with various concentrations of prostaglandin E₂ (Sigma Chemical Co., St. Louis, MO) and incubated in a CO₂ incubator at 37°C for specified time periods. Upon completion of incubation periods, culture supernatants were recovered and centrifuged, resulting in a cell-free supernatant to be tested by ELISA.

ELISA

The generation of anti-TNF receptor rabbit sera, purification of anti-TNF receptor rabbit IgG, labeling of anti-TNF receptor rabbit IgG with horseradish peroxidase, and specificity testing were performed utilizing methods described (18–21). Recombinant 55- and 75-kDa TNF receptors (22) used to generate antibodies, as well as to generate ELISA, were kindly supplied by Dr. Tadahiko Kohno of (Amgen, Inc., Thousand Oaks, CA). Briefly, anti-TNF receptor IgG (5 µg/ml in 0.05 M sodium bicarbonate buffer, pH 9.5) was added to each well of a 96-well, flat-bottomed, polystyrene ELISA plate (Corning Glass Works). Following an overnight incubation at 4°C, the wells were individually washed with 0.2% Tween-20 (Bio-Rad Laboratories, Hercules, CA) in phosphate-buffered saline (PBS). Recombinant TNF receptor standards and samples were added in duplicate to the wells. The plates were incubated at 37°C for 3 hr, after which another washing occurred. Horseradish peroxidase-labeled anti-TNF receptor IgG was added to each well, and plates were incubated for 1 hr in 37°C. The substrate, consisting of Immunopure ABTS tablets (2,2'-azinobis [3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt) (Pierce, Rockford IL), hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ), and 0.1 M sodium acetate buffer, pH 4.2, was added, followed by room temperature incubation for 20 min. Results were obtained with the use of an EAR 400 AT plate reader measuring OD₄₀₅ (SLT-Lab Instruments, Salzburg, Austria). Soluble receptor concentrations were calculated using the regression line derived from the known standards for each receptor. This assay is not able to distinguish between TNF/TNF-R complex and soluble TNF-R. TNF ELISA was also utilized, the method for which was identical to TNF-R ELISA, and also cannot differentiate between TNF/TNF-R complex and TNF.

Iodination of rTNF-α and Binding Assay

The iodination of rTNF-α was performed as described by Bolton *et al.* (23). One hundred microgram of rTNF-α (Genentech, South San Francisco, CA) in 20 µl of 0.1 M sodium borate buffer, pH 8.5, was added to the dried iodinated ester and the reaction mixture (Bolton-Hunter Reagent, Monoiodo, ¹²⁵I, DuPont, Wilmington, DE). Free iodine was removed by gel filtration over a NAP-5 column (Pharmacia, Sweden) with PBS containing 5 mg gelatin. One hundred fifty-microliter fractions were collected and 1-µl aliquots were assayed by

Clinnigamma Counter (Pharmacia, LKB, Uppsala, Sweden). Peak fractions were pooled and stored at 4°C to be used within 1 month.

The procedure for TNF binding assay analysis was performed as described by Gatanaga *et al.* (24). One million cells were incubated at 37°C for 6 hr in 1 ml of binding buffer (RPMI 1640 + 10% FBS), with or without 10^{-7} M PGE₂ in 24-well tissue culture plates. The cells were then incubated with 1 to 30 ng of ¹²⁵I-labeled TNF- α , in the presence or absence of a 100-fold excess of unlabeled TNF- α . Following a 3- to 4-hr incubation at 4°C, the buffer was decanted and cells were washed two to four times with ice-cold PBS (25). The cells were then solubilized in 1 ml of 1 N NaOH and radioactivity was determined by Clinnigamma Counter. The number of membrane TNF- α receptors and measure of their affinity were calculated by Scatchard plot analysis.

RNA Purification/Isolation and Analysis by Northern Blot

Total cellular RNA was isolated from THP-1 cells via the guanidinium isothiocyanate/cesium chloride method of purification. The RNA was quantitated spectrophotometrically at wavelength 260. Ten microliters of RNA was electrophoresed in 2.2 M formaldehyde in 1.2% agarose gels. The RNA is then transferred to nitrocellulose filter (Schleicher & Schuell, Keene, NH) and subsequently baked at 80°C for 2 hr. The filters were then hybridized with ³²P-labeled TNF and TNF receptor specific synthetic probes, as well as with a β -actin control probe. Following the hybridization, the filters were washed at 25°C with 2 $\times$ SSC, 0.1% SDS for 15 min twice and then with 0.1 $\times$ SSC, 0.1% SDS for 15 min. The filters were exposed to X-Omat XAR-5 (Kodak, Rochester, NY) X-ray films at -70°C. Band intensities were confirmed by analysis via gel digitizer (UVP Image Storage-5000) and quantitatively compared to those of β -actin, as well as to bands receiving no treatment.

Inhibition Assays

Several assays were performed to determine the action of inhibitors of certain pathways. One millimolar concentrations of Pefabloc (Boehringer Mannheim, Indianapolis, IN), a known serine protease inhibitor, were added to THP-1 cells with and without PGE₂. The cells were incubated in 37°C for a period of 6 hr, after which the levels of TNF receptors were measured by ELISA.

Similarly, the THP-1 cells were subjected to incubation with *N*-ethylmaleimide (NEM) (Sigma Chemical Co., St. Louis, MO), a known inhibitor of cell membrane internalization, again with and without PGE₂. Incubation of THP-1 cells in low temperature (16°C) is also known to exert the same inhibition of internalization and was utilized as well. All culture supernatants were centrifuged and subsequently analyzed by ELISA.

FIG. 1. Regulation of TNF and soluble 55- and 75-kDa TNF receptors in cell-free supernatants of THP-1 cells stimulated with PGE₂. 1×10^6 THP-1 cells were seeded in 24-well tissue culture plates and incubated for 6 hr with 10^{-7} M PGE₂. Cell-free supernatants were subsequently tested by ELISA. The effect of PGE₂ on soluble TNF receptor release was significant (55-kDa TNF-R, $P < 0.05$; 75-kDa TNF-R, $P < 0.01$; TNF, $P < 0.05$).

RESULTS

Regulation of TNF and TNF Receptor Release from PGE₂-Activated THP-1 Cells in Vitro

One million THP-1 cells were cultured per well with 10^{-7} M PGE₂ for 6 hr at 37°C in a CO₂ incubator. Cell-free supernatants were assayed for TNF, as well as for 55- and 75-kDa TNF receptors by ELISA. Over four trials, the average TNF concentration for the cells incubated in media was 229 pg/ml, while the mean for cells in PGE₂ was 161 pg/ml. Thus results show an average $21 \pm 8.8\%$ down regulation of the release of TNF (Fig. 1). PGE₂ suppression of TNF release in THP-1 cells is consistent with prior findings (17). In contrast, detection of the soluble forms of the TNF receptors revealed a general increase compared to spontaneous release control levels. Fifty-five-kilodalton receptor release increased an average $12.25 \pm 5.1\%$, from 104 to 122 pg/ml, whereas 75-kDa receptor release increased $55.5 \pm 18\%$, from 199 to 305 pg/ml at the same PGE₂ concentration (Fig. 1). All values were significant by Student's *t* test ($P < 0.05$). PGE₂ activation of THP-1 cells at 10^{-6}

(B)

	# of Receptors/Cell	Kd
Control	1190	3.07×10^{-10} M
PGE ₂	2533	2.29×10^{-10} M

FIG. 2. The effect of PGE₂ on the expression of TNF- α membrane receptors in THP-1 cells. 1×10^6 cells were incubated at 37°C for 6 hr in binding buffer with or without 10^{-7} M PGE₂ in 24-well tissue culture plates. They were then incubated at 4°C for 3–4 hr with 1 to 30 ng of ¹²⁵I-labeled TNF- α , in the presence or absence of a 100-fold excess of unlabeled TNF- α . (A) ¹²⁵I-labeled TNF- α binding on PGE₂-treated (□) or untreated (○) cells. (Inset) Scatchard analysis of the equilibrium binding data. (B) TNF receptor number and affinity comparison of PGE₂-treated and untreated cells.

and 10^{-8} M concentrations was also investigated, as well as the effect of a shorter 2-hr stimulation period. Both variations resulted in the same general trend as was found with 6-hr, 10^{-7} M PGE₂ treatment (data not shown).

PGE₂ Regulation of Membrane TNF Receptors

To determine the specific regions of PGE₂ regulation in THP-1 cells, the expression of cell surface receptors was examined. THP-1 cells were cultured in the same

manner as described above and incubated in a CO₂ incubator. The cells were subsequently cultured with ¹²⁵I-labeled TNF, with or without an excess amount of cold TNF, and analyzed via binding assay. Activation by PGE₂ for 6 hr resulted in a greater than twofold increase in the number of membrane-bound receptors (Fig. 2). It was determined through Scatchard analysis that the control group had 1190 surface receptors per cell, whereas a 6-hr incubation with PGE₂ yielded 2533 receptors per cell. Affinity constants remained comparable within groups as the control affinity was $3.07 \times$

10^{-10} M and for PGE₂ was 2.29×10^{-10} M. Again, a shorter activation period of 2 hr was also investigated, and the results indicate a similar increase of cell surface receptors (data not shown).

Northern Blot Analysis of PGE₂ Regulatory Effects of TNF and TNF Receptors

THP-1 cells were stimulated with PGE₂ for a period of 6 hr in 37°C, CO₂ incubation and then subjected to RNA purification methods and Northern hybridization. Resulting gels were then analyzed through use of a gel digitizer (UVP, Image Storage-5000, Ultra-Violet Products, San Gabriel, CA). RNA band densities of PGE₂-stimulated cells were digitally compared to β -actin mRNA levels, as well as additionally to mRNA of THP-1 cells receiving no treatment. As RNA band intensities in PGE₂-treated cells indicate, the level of 55-kDa receptor was nearly identical to the β -actin levels (7% decrease compared to control). Seventy-five-kilodalton mRNA levels in PGE₂-treated cells were shown to increase by 0.5% of the β -actin control density (Fig. 3). Results indicate no significant regulation of 55- or 75-kDa receptors by PGE₂ at mRNA transcription levels.

Inhibition of Serine Protease Mechanism by Pefabloc

It has been proposed by Hwang *et al.* (27) that the extracellular domains of the 55- and 75-kDa receptors are cleaved in THP-1 cells by action of a serine protease to yield the soluble forms of the receptors. In order to establish the mechanism through which PGE₂-modulated receptor release occurs, serine proteases are inhibited. Pefabloc, an analog of PMSF, is also a serine protease inhibitor and is utilized to test whether PGE₂-stimulated receptor release occurs by the same pathway as does spontaneous release. One millimolar amounts of Pefabloc were administered to THP-1 cells and followed by a 6-hr incubation in 37°C, after which soluble receptor levels were tested by ELISA. Cells treated with Pefabloc alone demonstrated depressed receptor numbers. For instance, the 55-kDa receptor was detected at a 59% decrease compared to the spontaneous release amount (Fig. 4A), and the 75-kDa receptor decreased 78% (Fig. 4B). PGE₂-treated cells showed an upregulation of receptors, the 55-kDa receptors increasing by 92% and the 75-kDa receptors increasing

	55 kD	75 kD
% Increase/Decrease	-7 %	0.5 %

FIG. 3. The effect of PGE₂ on TNF receptor mRNA levels in THP-1 cells. Total RNA was extracted from THP-1 cells following a 6-hr stimulation by PGE₂, as well as from cells receiving no treatment. 10 μ g of each total mRNA was applied to Northern blot analysis. Band intensities correspond to RNA amounts which were quantitated through use of a gel densitometer.

FIG. 4. Inhibition of soluble TNF receptor release from PGE₂-stimulated THP-1 cells by Pefabloc. 1×10^{-6} THP-1 cells were incubated at 37°C for 6 hr in 10^{-7} MPGE₂ with or without 1 mM Pefabloc. The amounts of soluble TNF receptors in cell-free supernatants were assayed by ELISA. (A) Level of soluble 55-kDa TNF receptor. (B) Level of soluble 75-kDa receptor.

by 169%. Pefabloc applied concurrently with PGE₂ resulted in a depression of receptor numbers comparable to that exhibited with Pefabloc alone (244 ± 2.4 versus 550 ± 61 pg/ml for 55 kDa, and 48 ± 2.2 versus 265 ± 7.1 pg/ml for 75 kDa).

NEM and Low-Temperature Inhibition of Internalization

Although cleavage by serine proteases has been implicated in TNF receptor shedding, general agreement on the exact location or pathway of receptor prior to shedding has not yet been established. A proposed route through which receptors venture involves a process in which cell surface receptors internalize into the cell membrane and then become cleaved by action of serine protease. In order to establish whether internal-

ization is an integral process in PGE₂-mediated receptor release, THP-1 cells were incubated with 1 mM NEM, an internalization inhibitor, for a period of 6 hr in 37°C. The cell supernatants were then analyzed for soluble receptor levels by ELISA. Receptor level fluctuations were similar to that seen with Pefabloc treatment. Cells treated solely with NEM exhibited considerable downregulation of soluble TNF receptor levels when tested by ELISA, compared to spontaneous release levels. Seventy-five-kilodalton receptor levels in cells treated with NEM alone were a mere 10% of spontaneous release amounts (Fig. 5B), whereas 55-kDa receptor levels with NEM comprised only 16% of control levels (Fig. 5A). Contrastingly, stimulation with PGE₂ elicited a 148% increase in 55-kDa TNF receptor levels and a 144% increase in 75-kDa levels. THP-1 cells stimulated simultaneously with PGE₂ and NEM produced a downregulation in receptor release, one which is comparable to that found with NEM alone (85% for 55 kDa and 88% for 75 kDa).

FIG. 5. The effect of NEM on the release of soluble TNF receptors from THP-1 cells stimulated by PGE₂. 1×10^{-6} THP-1 cells were incubated at 37°C.

Low-temperature incubation of THP-1 cells produced results similar to those of NEM stimulation. Incubation (16°C) yielded soluble 55-kDa TNF receptor levels of 1165 pg/ml, a 298% increase compared to its low-temperature counterpart which produced only 293 pg/ml. The amount of soluble 75-kDa TNF receptors increased by 192% in the cells incubated in normal temperature (37°C), as opposed to those treated with low temperature.

DISCUSSION

PGE₂ has been found to control TNF activity via two modes: (i) Direct suppression of the TNF molecule (16), and (ii) upregulation of TNF receptors which may in turn bind to and subsequently regulate TNF activity (14). This study not only supports the theory of the integration of neuroendocrine and immune systems, but additionally, it provides evidence of a new pathway of regulating the wide range of immune functions in which TNF is involved. PGE₂ is a key inhibitor of tumor necrosis factor at both the transcriptional and the post-transcriptional levels (17). This suppression occurs in a dose-dependent manner and is not limited solely to the restriction of TNF, as interleukin-1 represents another cytokine regulated by PGE₂ (26). PGE₂ additionally exerts a mechanism of feedback inhibition upon TNF production, observed as TNF stimulation of fibroblasts and monocytes results in a decreased production of PGE₂ (26). The regulation of TNF production and release makes PGE₂ a significant immunomodulatory factor, an important link, so to speak, in the network of molecules and hormones bridging the immune and neuroendocrine systems together.

PGE₂ regulation of TNF in THP-1 cells has been established in several studies (16, 17). However, further evaluation revealed regulation of soluble TNF receptor levels with PGE₂ treatment as well. We report here that both 55- and 75-kDa soluble receptor levels increased dramatically with PGE₂ activation. This treatment, furthermore, increased the number of membrane-bound receptors by more than two-fold, with no significant change in affinity. This increase in cell surface and soluble receptor expression prompts us to expect a comparable upregulation at the protein transcription level. Interestingly, TNF receptor mRNA levels, as analyzed by Northern hybridization, remain unchanged within control groups and those which were activated by PGE₂. These findings suggest the existence of an intracellular pool of TNF receptors situated in the region between transcriptional activities and cell surface expression. The existence of such would account for the variation in surface receptors while the mRNA levels remain unchanged. Yet another possibility for the discrepancy may be a greater translation of receptors than that detectable at the mRNA transcription level.

Prior findings indicate that serine protease is respon-

sible for both spontaneous and PMA-induced shedding of the soluble forms of TNF receptors from THP-1 cells *in vitro* (27). In an attempt to elucidate whether regulation by PGE₂ occurs by an analogous pathway, Pefabloc, an analog of PMSF, a known serine protease inhibitor, was implemented alone, as well as concurrently with PGE₂. Pefabloc successfully blocked spontaneous release levels of both soluble 55- and 75-kDa receptors. Furthermore, Pefabloc attenuated PGE₂ enhancement of receptor release, indicating that serine protease may comprise a vital component in the pathway for spontaneous receptor release, as well as PGE₂-mediated release.

The exact mechanism of action by serine proteases is not clear, however, especially concerning the specific locale of action. It is not known whether the receptor cleavage occurs intracellularly before initial contact with the cell membrane or whether receptors are shed from the membrane postinternalization. Yet another possible mechanism involves cleavage of the receptor at the cell surface (27). Inhibitors of internalization were therefore utilized alone and in conjunction with PGE₂ in order to narrow the modes of action. Low-temperature incubation and NEM, both known internalization inhibitors, downregulated soluble receptor levels when administered alone. NEM also blocked the PGE₂ increase of soluble receptors to a level comparable to that achieved with NEM blockage of spontaneous release. Therefore, a viable possibility is that internalization of membrane receptors is an integral factor prior to release in PGE₂-activated cells. But at the same time, these data do not eliminate the likelihood of TNF receptor regulation by way of enzyme mobility inhibition. As NEM blocks movement of the receptor through the cell membrane, through parallel action, it may also inhibit movement of the serine protease and thus interfere with receptor shedding.

The role of PGE₂ in regulation of TNF and its receptors is reciprocated by TNF regulation of PGE₂ as well. These findings contribute to recent findings which have evolved to establish a bidirectional communication between systems. It is postulated that the immune system, therefore, may serve as a sensory organ, sensing stimuli which fail to be recognized by the central or peripheral nervous systems (15). On the same note, it has been established that nervous system recognition of stimuli can also be converted into chemical signals which can then be relayed to immune cells, eliciting a physiological response. This interactive network allows for the opening of many possibilities as far as hormonal manipulation of the immune system. The options involved in clinical treatment of immune disorders may thus be greatly expanded.

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