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Demonstration of the presence of an interleukin-1 receptor on the surface of murine astrocytes and its regulation by cytokines and Theiler's virus

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SUMMARY

Interleukin-1 (IL-1) binding found in rodent brain, and its biological effect on astroglial cells, suggests that the receptor for IL-1α may be located on astrocytes. A radioligand receptor assay performed on pure mouse astrocyte cultures revealed the presence of one binding site [type I, molecular weight (MW) 80,000], with $K_d = 0{\cdot}3 \times 10^{-10}$ M, at a density of 150 receptors/cell. The specific, saturable and displaceable high-affinity binding took place in a time- and temperature-dependent way. The receptor–IL-1α complex was quickly endocytosed at 37° but not at 4°. The receptor expression was up-regulated by Theiler's virus, interferon-γ, tumour necrosis factor and IL-6; was not affected by vaccinia virus infection; and decreased after lipopolysaccharide treatment.

INTRODUCTION

Interleukin-1 (IL-1) is composed of a family of proteins, mainly produced by activated macrophages, that was first defined as a T-lymphocyte mitogen.[1,2] Further studies demonstrated that this cytokine possesses a great number of biological activities, including some related to the central nervous system (CNS), such as astroglial growth,[3] fever induction,[4] slow wave sleep[5] and appetite control.[6] Its role as a CNS inflammatory mediator has been documented, i.e. glial cells produce IL-1 upon stimulation with lipopolysaccharide (LPS),[7] and SJL/J astrocytes synthesize it when specifically infected with the neurotropic Theiler's murine encephalomyelitis virus (TMEV).[8] Furthermore, the CNS of demyelinated mice contains measurable levels of IL-1α detected by specific radioimmunoassay (RIA).[9]

Two forms of IL-1 receptor encoded by separate genes, IL-1R type I and type II, have been identified.[10,11] The fact that IL-1 receptors have been visualized throughout the rat brain by autoradiography[12,13] and that its mRNA has been detected in the brain of BALB/c mice[14] (mainly in its dentate gyrus) suggested the study of the presence of IL-1α receptors on the surface of pure murine astrocyte cultures.

Therefore, this study addressed the question of whether there are specific IL-1 receptors on astrocytes by performing radioligand binding assays on purified astrocytes maintained in tissue culture. The data presented in this article show that there are indeed specific IL-1α receptors of type I [80,000 molecular weight (MW)] displaying classical characteristics of a protein receptor, i.e. saturable and reversible binding, high affinity and specificity for the ligand.

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MATERIALS AND METHODS

Mice

SJL/J and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained on standard laboratory feed and water *ad libitum* in the Instituto Cajal, Madrid, Spain, animal care facilities.

Astrocyte cultures

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn BALB/c or SJL/J mice.[15] The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through 80-μm pore size mesh into Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and gentamicin (Flow Laboratories, Irvine, U.K.). After centrifugation, cells were filtered through a 20-μm mesh sieve, plated in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) and cultured at 37°. The medium was changed after 4 days of culture and, subsequently, three times a week for the entire culture period. Cultures were enriched in astrocytes by removal of less adherent oligodendrocytes by shaking for 18 hr at 37° and 250 r.p.m. in a G24 environmental shaker (New Brunswick Scientific, Edison, NJ). Cellular confluence was observed 10 days after plating, with a polygonal flat cell morphology. A mean of 98·4% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillar acidic protein (GFAP) antiserum (Dakopatts, Glostrup, Denmark) and fluorescein-labelled goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, IN). The lack of noticeable oligodendrocytes and microglial/macrophage cells

was determined using a guinea-pig anti-myelin basic protein (MBP) antiserum prepared as described elsewhere[16] and a monoclonal anti-Mac-1 antibody (Serotec, Oxford, U.K.). Secondary fluorescein-labelled antibodies against guinea-pig and rat IgG were purchased from Sigma Chemical Co. (St Louis, MO).

The rat glioma C-6 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM supplemented with 10% FCS and gentamicin.

Binding assay

Confluent astrocyte monolayers in 16-mm diameter 24-well plates (Costar) were used in the binding experiments. Cells were incubated at different temperatures and times with labelled IL-1α, as stated in the text. The medium used was basal Eagle's medium (Flow) containing 0·2% bovine serum albumin (BSA; Sigma). After washing three times with the above medium, cells were detached from the plastis surface with 2% SDS at 60° and counted in a LKB–Wallac 1282 Compugamma counter (Pharmacia, Uppsala, Sweden).

^{125}I-IL-1α (human, recombinant) was purchased from New England Nuclear–Dupont (Boston, MA) (NEX-246) with a specific activity of 107 μCi/μg.

Endocytosis

To assess total internalized ligand, the surface-bound ^{125}I-IL-1α was removed from twice-washed cells with 2 ml of 0·15 M NaCl–50 mM glycine, pH 3·0, at 4° for 2 min.[17] After two more washes, cells were detached with SDS and counted in the γ-counter.

Statistical analysis

Scatchard plot analysis of the data from binding assays was performed employing the LIGAND program.[18] The data shown represent mean results of three reproducible experiments ±SD

Astrocyte culture infection and cytokine treatment

Astrocytes were cultured in 24-well plates after trypsinization from 75 cm^2 tissue flasks. When reaching confluence (usually after 48 hr of subculture), cells were washed twice with 2 ml of complete DMEM and infected with 1 ml of 10-fold dilutions of virus or treated with cytokines or LPS. The treatment was allowed to take place overnight at 37° in a 10% CO_2 atmosphere. After three more washes, cells were tested for ^{125}I-IL-1α binding, as stated above.

Cross-linking to the IL-1α receptor

Confluent astrocyte cultures in 60-mm diameter Petri dishes were incubated for 18 hr at 4° with saturating amounts of ^{125}I-IL-1α. After three washings with culture medium, the cells were detached in ice-cold phosphate-buffered saline (PBS) with a rubber policeman, centrifuged in Eppendorf tubes and the pellets resuspended in 10 mM dimethyl pimelidate (Sigma) buffered in 0·2 M triethanolamine, pH 8·2. The samples were then incubated at room temperature in a vigorous shaking bath for 1 hr and stopped by addition of sample buffer. The material was boiled for 2 min and subjected to SDS–PAGE in a 7–17% gel gradient. Samples were normalized for protein content before gel loading. The optimum exposure time for the autoradiography when using enhancer screens was 3 days. Non-specific binding was determined in the presence of a 200-fold excess of cold IL-1α.

Interleukins and virus

Recombinant IL-1α of human origin was kindly provided by Dr Anthony Meager (National Institute for Biological Standards, Potters Bar, U.K.), and mouse rIL-1α was from Genzyme (Cambridge, MA). Recombinant mouse interferon-γ (IFN-γ) was purchased from Holland Biotechnology (Leiden, the Netherlands), and recombinant murine tumour necrosis factor-α (TNF-α) was purchased from Innogenetics (Antwerp, Belgium). Human rIL-6 was from Boehringer Mannheim (Mannheim, Germany) and *Escherichia coli* LPS were obtained from Sigma.

A strain of Theiler's murine encephalomyelitis virus (TMEV), isolated in 1957 from a feral mouse in Belem, Brazil (BeAn 8386), was used. Stocks of 10^7 plaque-forming units (PFU)/ml were prepared as described by Rozhon *et al.*[19] Virions were purified by isopyonic centrifugation in caesium chloride gradients.[20] Vaccinia virus stocks at 200 × 10^6 PFU/ml were kindly provided by Dr Eduardo Paez (Centro de Investigaciones Biologicas, Madrid, Spain).

RESULTS

Kinetics of binding at 37° and 4°

The binding of ^{125}I-IL-1α to its cell receptor on the surface of SJL/J and BALB/c astrocytes is shown in Fig. 1. At 37° the binding to BALB/c astrocytes reached saturation after a period of 60 min. For SJL/J, the kinetics was somewhat slower, reaching maximum binding after 120 min of incubation. At 4°, the steady state reached about 50% of the maximum value obtained at 37°, independent of the strain of mice used. This relative temperature dependence of the binding of IL-1 has been described previously in sections of rat brain by Farrar *et al.*[13] As no differences between strains were found at 37° after a period of incubation of 120 min, further binding experiments were all executed with BALB/c astrocytes under the above conditions, unless otherwise stated. Maximum binding was found to be around 4% of the input (100,000 c.p.m./well).

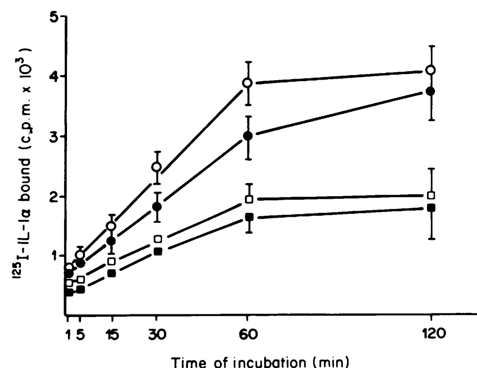

Figure 1. ^{125}I-IL-1α binding kinetics of BALB/c (○) and SJL/J (●) astrocytes at 37°. The time-course at 4° is also shown for BALB/c (□) and SJL/J (■) glial cells. Each data point represents the mean of three separate experiments ± SD, in all the figures.

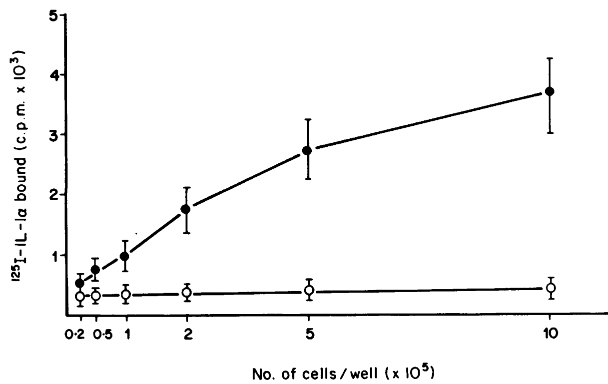

Figure 2. Effect of cell concentration in the total binding of ^{125}I-IL-1α. The indicated numbers of astrocytes (●) were incubated with 100,000 c.p.m. of ^{125}I-IL-1α for 60 min at 37°. The lack of binding of C-6 glioma cells (○) is also shown.

Number of cells

Binding of IL-1α to astrocytes varied as a function of the number of cells seeded, reaching a maximum at 10^6 astrocytes from a well of 16 mm diameter (Fig. 2).

The rat glioma C-6 did not bind any IL-1 independently of the number of cells used, demonstrating the absence of cellular IL-1 receptors on its surface. The non-specific binding observed in the absence of cells reached values of $0{\cdot}4 \pm 0{\cdot}1\%$ of the input. The unlikely possibility of high-density IL-1 receptor expression on contaminating cells such as microglia was addressed by reducing cellular contamination in the cultures to undetectable levels.

Characterization of the IL-1 binding site

The specificity of the binding of IL-1α to its receptor is depicted in Fig. 3. Total and specific binding, defined as the binding which is inhibited in the presence of 200-fold excess of cold IL-1, are shown. The curve obtained indicates that the binding was saturable and inhibited by unlabelled IL-1 in a dose-dependent fashion.

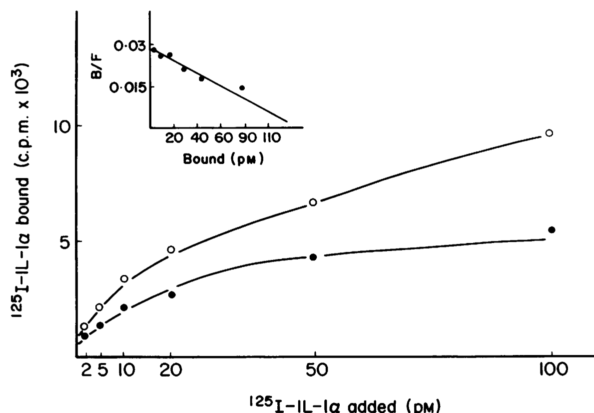

Figure 3. Specific (●) and total binding (○) of murine IL-1α to astrocytes, as a function of its concentration. The inset shows the plot of the data according to Scatchard analysis.

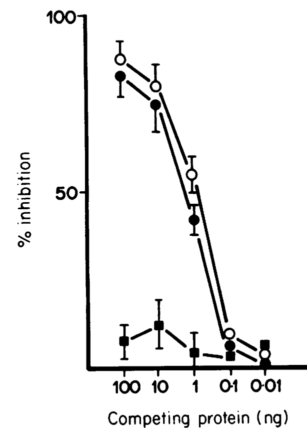

Figure 4. Species specificity of the binding of IL-1α to its receptor on the astrocytic cells. The binding of ^{125}I-IL-1α (100,000 c.p.m.) taking place in the presence of the indicated amounts of unlabelled murine rIL-1α (●), human rIL-1α (○) and murine rTNF-α (■) was plotted as the percentage of the inhibition obtained.

Scatchard plot analysis (Fig. 3, inset) using the LIGAND program[18,21] demonstrated the presence of only one type of binding site with a K_d of $0{\cdot}3 \times 10^{-10}$ M and an approximate number of 150 binding sites/cell. This surprisingly low number of receptors/cell has been described previously in the IL-1 system.[22] Half saturation was achieved at a concentration of free ligand of 20 pM.

Species specificity of the binding

As no mouse IL-1α labelled with ^{125}I was available from commercial sources at the time, human recombinant IL-1α was used in the above experiments. Kilian *et al.*[23] previously reported that human and mouse IL-1 behave equally for binding to the receptor of a murine T lymphoma. Hence, the ability of unlabelled mouse recombinant IL-1 to compete with human ^{125}I-labelled IL-1 for the astrocyte receptor was tested. As shown in Fig. 4, increasing concentrations of mouse or human competing proteins inhibited the binding of human ^{125}I-IL-1α in a similar way, demonstrating that both recombinant cytokines bound to the same receptor with the same affinity. Approximately 85% of the radioactivity was displaced by 100 ng of 'cold' cytokines, the remaining binding being considered as non-specific. Increasing amounts of mouse recombinant TNF were not able to bind to the IL-1 receptor on the astrocytes, demonstrating the specificity of the binding.

Internalization of IL-1

As shown in Fig. 5, ^{125}I-IL-1α was internalized by murine astrocytes with a half-time of maximal internalization of about 3 min. The internalized IL-1α (pH 3·0 resistant) increased with time, reaching a plateau after 60 min at 37°. No significant internalization took place at 4°, demonstrating an energy-dependent endocytosis of the bound cytokine, as described previously for the astrocytic receptor for IFN-γ.[24]

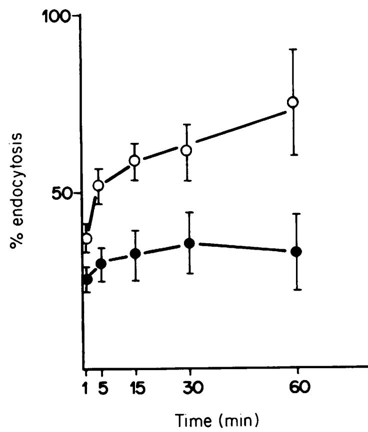

Figure 5. Internalization of IL-1α by mouse astrocytes. The kinetics of endocytosis are shown at both 37° (○) and 4° (●) as the percentage of c.p.m. internalized by the cells.

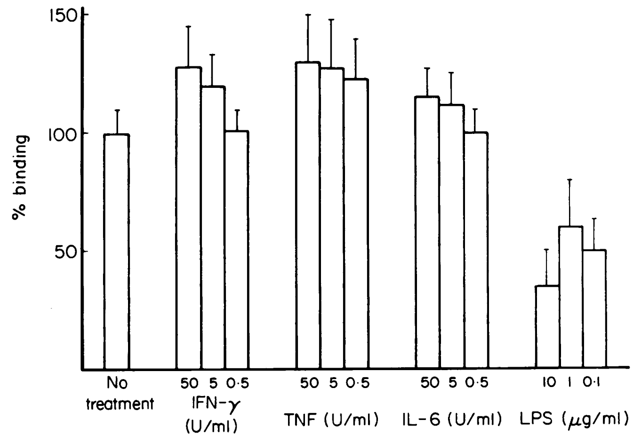

Figure 7. Changes in the expression of IL-1 receptor after an overnight treatment of astrocytes by different amounts of cytokines (IFN-γ, TNF-α and IL-6) or *E. coli* LPS. Untreated control cells were considered as having 100% binding.

Regulation of IL-1 receptor on astrocytes

Some stimuli related to inflammatory processes inside the CNS were investigated as possible factors of regulation of IL-1 receptor expression.

TMEV binds to murine astrocytes of both SJL/J and BALB/c strains, which bear 2500 viral receptors on their cell surface.[15] Furthermore, when infected by TMEV, astrocytes release several cytokines.[8,25,26] The astrocytic cultures in the present study had been infected with TMEV and vaccinia virus as negative control, and the amount of receptors for IL-1α was checked. As shown in Fig. 6, TMEV up-regulated the expression of IL-1 receptor in a dose-dependent manner. When a multiplicity of infection (MOI), ratio between the number of virus PFU and the number of cells, of 10 or 1 was used, the expression of the receptor reached values of 170% and 124% of the values found on mock-infected cells. A MOI of 0·1 and 0·01 did not stimulate any change in the expression. Scatchard plot analysis by the LIGAND program demonstrated that the increase in IL-1-binding levels corresponded to an increase in the number of receptors on the surface of the astrocytes, and not with changes in its K_d (not shown). Vaccinia virus, an unrelated DNA virus to which glial tumour cells such as C-6 are susceptible, did not change the expression of IL-1 receptors, independent of the number of virus PFU used for infection.

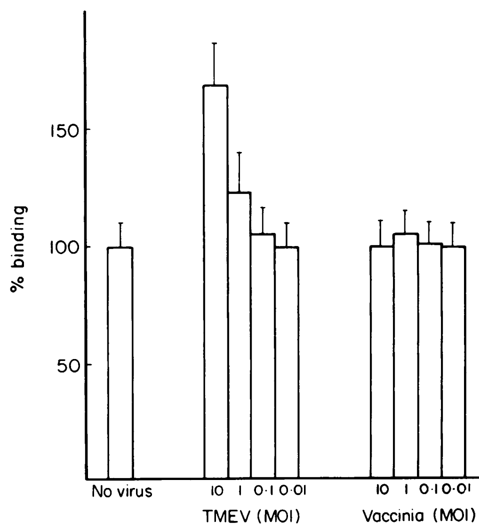

Figure 6. Changes in the expression of the IL-1 receptor induced by virus infection. Mock-infected control cells (no virus) or cells infected with decreasing MOI of TMEV or vaccinia virus were subsequently tested for ^{125}I-IL-1α binding.

As TNF and IL-6 act in a synergistic way with IL-1 in inflammatory processes, their possible effect on the regulation of the astrocytic IL-1 receptor was also studied. The effect of IFN-γ, for which the astrocytes have 11,100 receptors/cell[24] and which exerts a clear biological activity on astrocytes[27,28] was also investigated. All the three treatments with cytokines up-regulated the expression of the receptor in the range of 50–5 U/ml (Fig. 7). The increase was significant, reaching maximal increments of 30%. These changes corresponded, as for TMEV, to an increase in the number of receptors, maintaining the same affinity.

The treatment with the non-specific mitogen LPS dramatically decreased the availability of receptors on the surface of the cells. This was probably due to the cytopathic effect of the mitogen, which could even be observed under the microscope.

Affinity cross-linking of ^{125}I-IL-1α to its receptor

Astrocytes were incubated at 4° overnight with saturating amounts of ^{125}I-IL-1α, washed and cross-linked with 10 mM dimethyl pimelidate. The product was visualized on SDS–PAGE as a 97,000 MW band (Fig. 8A). Its formation was blocked in the presence of a 200-fold excess of unlabelled IL-1 (Fig. 8B). This result suggests a size of 80,000 MW for the astrocyte IL-1 receptor, as the IL-1α molecular weight is 17,500.[22] This size is in accordance with the previously described molecular weight of type I receptor in other cell types.[10,11]

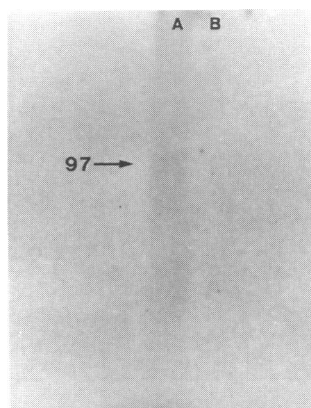

Figure 8. Cross-linking of ^{125}I-IL-1α to its receptor on astrocytic cell membranes. A band with an apparent molecular weight of 97,000 was obtained after autoradiography of the SDS–PAGE slab (A). The formation of that band was inhibited by a 200-fold excess of unlabelled IL-1α (B).

DISCUSSION

IL-1 mediates a broad array of systemic events including immunological, inflammatory and neurological effects. We were interested in the interaction of astrocytes with cytokines, and IL-1 produces important biological effects on this glial population. In fact, IL-1 induces a growth-promoting activity on astrocytes[3,28,29] and regulates the gene expression of proenkephalin[30] and nerve growth factor.[31]

All these pleiotropic effects must be exerted through specific binding of IL-1 to a plasma membrane receptor(s). Two different receptors for IL-1 have been described: type I (80,000 MW); and type II (60,000 MW).[10,11] Both act through a specific signal transduction system.[32]

In this article we studied the presence and mechanisms of expression of the only receptor for IL-1α on the membrane of mouse astrocytes. The binding was temperature-dependent and proportional to the number of cells used (Figs 1 and 2). Its binding affinity was estimated as $0{\cdot}3 \times 10^{-10}$ M and demonstrated as being specific and saturable (Fig. 3). The reported affinity agrees with that previously published for other type I receptors.[10] The low number of receptors reported (150/cell) has been detected previously in this system.[22] The demonstration of the presence of these receptors and their high affinity explains the biological activity exerted by IL-1 on astrocytes.

The receptor for IL-1α and IL-1β has been reported to be the same[23] and we demonstrate here that both mouse and human IL-1α bind equally to mouse astrocytes (Fig. 4). The molecular weight of the receptor, obtained by cross-linking experiments (Fig. 8), is reported to be 80,000, which corresponds to the type I receptor that is usually expressed on T-lymphocytes and fibroblasts.[10,11] The receptor–ligand complexes are usually endocytosed and processed intracellularly. We have studied the internalization of the astrocytic IL-1–receptor complex, which takes place at 37° but not at 4°, demonstrating that this is an energy-dependent process. The endocytosis is fast, with a maximal internalization half-time of 3 min (Fig. 5).

Although some reports about the induction of IL-1 in brain cultures by virus,[33] and particularly by TMEV in astrocytes,[8] have been released, we do not know of any previous reports of an increase of IL-1 receptors as a consequence of viral infection. TMEV induced such an increase in number, but not in affinity, of receptors for this ligand. This up-regulation is dose-dependent, being maximal when a MOI of 10 is used (Fig. 6). An unrelated DNA vaccinia virus did not change the amount of receptors on infected cells.

In a similar way, the cytokines that actively change the biology of astrocytes such as IFN-γ,[27] or that act synergistically with IL-1 in inflammatory processes (TNF and IL-6), up-regulated the expression of the receptor for IL-1α (Fig. 7). This must have important implications in the biology of TMEV-induced demyelination, as TMEV also induces the synthesis of both TNF[26] and IL-6.[25] Conversely, LPS treatment produced a three-fold reduction of the binding of IL-1. This mitogen induces the production of both IL-1 receptor antagonist and IL-1 in the same cells.[34] Nevertheless, we think that the low level of IL-1 binding is due to a cytopathic effect rather than to the release of any antagonist.

In a similar way, Theiler's virus induces both IL-1 production[8] and up-regulation of IL-1 receptor on BALB/c astrocytes, suggesting an autocrine pathway. Our results show that the demyelinating disease induced by TMEV probably results as a consequence of the action of several cytokines with synergistic effects. The conclusion is in accordance with the potentiation by TNF, IL-6 and IFN-γ of the number of IL-1 receptors on astrocytes.

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