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Substance P Augments Tumor Necrosis Factor Release in Human Monocyte-Derived Macrophages

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Substance P (SP) is an undecapeptide that has the amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and that belongs to a family of structurally related peptides known as tachykinins; the latter are widely distributed in the central nervous system. SP is involved in the biological activities of cells in the immune system, including the induction of cytokines in immune cells. We have investigated the effects of SP on constitutive and/or lipopolysaccharide (LPS)-induced expression of tumor necrosis factor (TNF) in cultured blood monocyte-derived macrophages (MDM). Cells cultured in vitro for 14 days were treated with SP at various concentrations (10^{-10} to 10^{-6} M) in the presence or absence of LPS before culture supernatants were harvested. TNF bioactivity in culture supernatants was measured with the L929 cell line. MDM from 10 of 12 donors treated with SP alone showed increased TNF production. SP and LPS also interacted in a synergistic fashion in upregulating TNF production in MDM from responders. The stimulatory effect of SP was inhibited by two SP antagonists, spantide ([D-Arg-1–D-Trp-7–D-Trp-9–Leu-11]-SP) and CP-96,345 (a nonpeptide antagonist of the SP receptor). In addition, an anti-SP polyclonal antibody blocked the SP effect on TNF production in cultured MDM, further indicating the specificity of these effects. These results demonstrate that SP is an important regulator of monokine production by human monocytes/macrophages.

Monocytes are a heterogeneous population of blood-borne cells, that are generally derived from bone marrow and that differentiate into macrophages upon appropriate stimulation. Macrophages regulate many aspects of immune function, including antigen processing, T-cell proliferation, lymphokine and antibody production, and antineoplastic defense (1).

Substance P (SP), the best known member of the tachykinin family, is a neurotransmitter involved in the conduction of nociceptive stimuli and a modulator of neuroimmunoregulation. SP is an undecapeptide that has the amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and that belongs to the tachykinins, a family of bioactive peptides sharing similar pharmacological properties and carboxyl-terminal sequences. SP is widely distributed in the central and peripheral nervous systems. It is released from nonmyelinated sensory nerve fibers, may accumulate at sites of inflammation, induces vasodilatation and plasma extravasation, and stimulates leukocyte chemotaxis (21, 24, 27). SP also evokes a number of responses from monocytes/macrophages, including the generation of thromboxane A2 and superoxide, the down-regulation of membrane-associated 5′-nucleotidase, and the stimulation of the synthesis and release of arachidonic acid and its metabolites (7, 8). Lucey et al. have demonstrated SP receptors on in vitro-cultured human blood-derived monocytes/macrophages (17). SP stimulates human peripheral blood monocytes to produce inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF), which are important constituents of immune cell activation that act as physiological inductive signals in the regulation of immune responses (15, 16). TNF alpha (TNF-α) was first described as a tumor toxin and has recently been recognized as an important mediator of inflammation (4); it upregulates human immunodeficiency virus type 1 expression in T cells and monocytes in vitro (12, 26). Whether SP directly induces macrophages to produce cytokines is unknown (15).

The effect of SP on TNF production by macrophages derived from human peripheral blood monocytes (MDM) has not been investigated. In the present study, we describe experiments designed to evaluate the effect of SP on TNF production by MDM. We show that SP modulates TNF production in MDM. These findings further support the concept that neuropeptides such as SP are important regulators of cytokine expression in MDM and further demonstrate the important link between the nervous system and the immune system.

MATERIALS AND METHODS

Isolation and culturing of monocytes/macrophages. Monocytes were purified by our previously described technique (10, 11). In brief, heparinized blood from a normal healthy donor was separated by centrifugation over lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.) at 400 to 500 × *g* for 45 min. The mononuclear cell layer was collected and incubated with Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) in a gelatin-coated flask for 45 min at 37°C; nonadherent cells were washed off with DMEM. Monocytes were detached with EDTA. Cells were plated in 24-well culture plates at a density of 5×10^5 cells per well or in 48-well culture plates at a density of 2.5×10^5 cells per well in DMEM containing 20% fetal calf serum. Following the initial purification, >97% of the cells were found to be monocytes by nonspecific esterase staining and fluorescence-activated cell sorting analysis with a monoclonal antibody against CD14 (Leu-M3) (10) and low-density

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lipoprotein specific for monocytes/macrophages (13). Cell viability was monitored by trypan blue exclusion and the presence of adherent cells.

SP treatment. SP and related peptides (Peninsula Laboratories, Belmont, Calif.) were dissolved in sterile distilled water to yield a stock solution of 10^{-3} M. The stocks of SP and related peptides were divided into aliquots in polypropylene microcentrifuge tubes (catalog no. 05-407-10; Fisher Scientific, Pittsburgh, Pa.) and kept at −80°C. The stocks were thawed at the time of the experiments and diluted in serum- and protein-free medium (catalog no. s-2897; Sigma Chemical Co., St. Louis, Mo.) before each experiment. Fourteen days after in vitro culturing, MDM were washed once with serum- and protein-free medium and treated with concentrations of SP that ranged from 10^{-10} to 10^{-6} M and that were diluted in the serum- and protein-free medium. Supernatants were collected at several times post-SP treatment and stored at −80°C until the assay. Truncated fragments of SP, i.e., SP^{1-4} and SP^{5-11}, were also tested. In some experiments, SP effects were examined with lipopolysaccharide (LPS)-stimulated macrophages. For cell-associated TNF, SP-treated or nontreated cells were washed once with fresh serum-free medium and harvested by repeated freezing-thawing. The cell debris was clarified by centrifugation, and supernatants were collected and stored at −80°C until use. Concentrations of SP as high as 10^{-4} M had no effect on MDM morphology, as evaluated by phase-contrast microscopy or by cell adherence.

Specificity of the effects of SP. To confirm the specific effects of SP, two SP antagonists, CP-96,345 (a gift from John G. Stam) and spantide ([D-Arg-1–D-Trp-7–D-Trp-9–Leu-11]-SP) (obtained from Peninsula Laboratories), were used. Anti-human SP rabbit serum and normal rabbit serum (Peninsula Laboratories) were also used. Some experiments were performed in the presence of polymyxin B sulfate (10 μg/ml; Sigma) to exclude the possibility that the SP preparations were contaminated with endotoxin that could induce TNF production in MDM.

Measurement of TNF secretion. TNF bioactivity in MDM supernatants was detected with an L929 (a murine cell line) bioassay as described previously (6, 14). In brief, confluent monolayers of L929 fibroblasts were trypsinized and resuspended at 3×10^5 cells per ml, and aliquots of 100 μl of the cell suspension were placed in each well of 96-well plates (flat bottom) and incubated in DMEM with 20% fetal calf serum at 37°C. Supernatants were removed 18 h posttreatment, and the cells were washed once with serum- and protein-free medium. Fifty microliters of actinomycin, D-mannitol (4 μg/ml), and 50 μl of a supernatant sample, TNF-α (a standard used as a positive control), or DMEM (used as a negative control) were added to each well and incubated at 37°C for 24 h. LPS or SP alone at different concentrations was also added to L929 cells as a reagent control. All supernatants were tested in triplicate. Cells were stained with crystal violet for 10 min and subsequently washed. The absorbance was measured with an automated microplate reader (model 2550; Bio-Rad Laboratories, Life Science Group, Melville, N.Y.) at 570 nm. TNF cytotoxicity was expressed as percent cytotoxicity as follows: percent cytotoxicity = (1 − absorbance of the sample/absorbance of the control) × 100.

RESULTS

TNF secretion in SP-treated MDM. The culture supernatants of primary monocytes/macrophages were collected every 48 h up to day 14. The highest concentrations of TNF were detected during the first 72 h and then declined steadily in the subsequent days. By week 2, TNF biological activity was not detectable in MDM cultures with the L929 cytotoxicity assay (Fig. 1). However, when MDM cultured for 14 days were treated with SP (10^{-6} M), TNF production was observed (Fig. 1). TNF production in a dose-dependent manner was also observed in cells treated with various concentrations of SP (Fig. 2). To determine which portion of the SP molecule mediates the increase in TNF production, the capacities of the N-terminal peptide SP^{1-4} and the C-terminal peptide SP^{5-11} to affect TNF production in MDM were evaluated. As shown in Fig. 3, SP^{sar} and truncated SP^{1-4} and SP^{5-11} induced TNF production in MDM, but the effects of the truncated SP molecules were less potent. SP (10^{-6} M) or LPS (10 ng/ml) added to L929 cells alone did not have a detectable cytotoxicity effect, as determined by a trypan blue dye exclusion assay (data not shown).

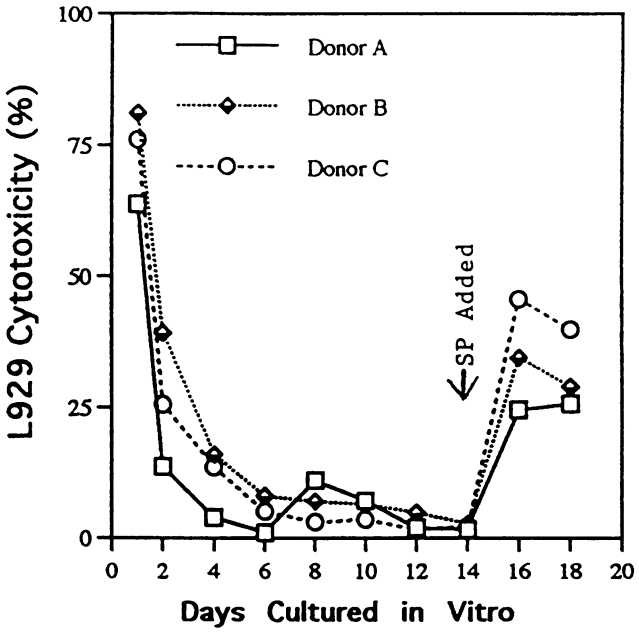

FIG. 1. Time course of TNF production by primary human monocytes/macrophages obtained from three different donors and cultured in vitro. The monocytes/macrophages were cultured in 48-well plates (2×10^5 cells per well) in the absence of LPS. SP (10^{-6} M) was added to the cells at day 14 of in vitro culturing. The production of TNF in culture supernatants harvested at the times indicated was determined with an L929 cytotoxicity assay. The results shown are means for triplicate cultures.

SP antagonists and an anti-SP antibody block SP effects. To evaluate the specificity of SP effects, two different SP antagonists, spantide (a pan-neurokinin receptor antagonist) and CP-96,345 (a nonpeptide antagonist that specifically binds to the NK-1 receptor), were tested. Control conditions included cells cultured in medium alone, in medium with SP alone, in medium with CP-96,345 alone, or in medium with spantide alone. The stimulatory effect of SP (10^{-6} M) in the presence of either spantide or CP-96,345 was reduced to control levels in MDM cultures, as measured by the L929 cytotoxicity assay (Fig. 4). These two antagonists added to MDM individually had no effect on TNF production (data not shown). In addition, an anti-human SP polyclonal antibody (rabbit serum, 1:200 dilution) blocked the stimulatory effect of SP on the production of TNF in MDM cultures, whereas normal rabbit serum had no effect on SP-induced TNF production (Fig. 4).

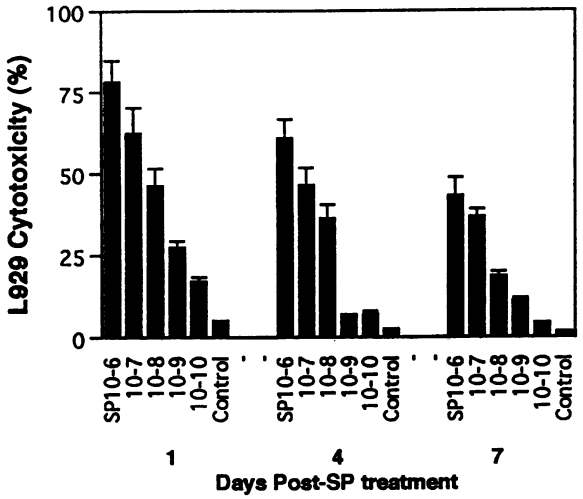

FIG. 2. TNF production in MDM treated with SP at various concentrations. MDM cultured for 14 days were treated with SP at the indicated concentrations. Supernatants were collected at days 1, 4, and 7 after SP treatment and examined for TNF production with an L929 cytotoxicity assay. The results shown are means for triplicate cultures, representative of two donors.

Anti-TNF-α and anti-TNF-β antibodies neutralize SP-induced MDM supernatants. Since the L929 bioassay cannot distinguish between TNF-α and TNF beta (TNF-β), antibodies to these two cytokines were evaluated for their ability to neutralize the cytotoxic effects of SP-induced MDM supernatants on L929 cells. As expected, antibodies to both TNF-α and TNF-β were able to neutralize the cytotoxic effects of the supernatants on L929 cells, while negative control serum (normal rabbit serum) did not have this effect (Fig. 4).

Donor variability. Donor variability of TNF production in SP-treated MDM from 12 different donors was observed (Fig. 5). Because MDM from 2 (donors 8 and 11) of the 12 donors studied showed no response to SP treatment (Fig. 5), we determined whether SP has synergistic effects with LPS on cells from these two donors. However, when SP and LPS were added to the MDM cultures, no synergistic effects were observed in these non-SP-responsive donors, although LPS alone stimulated TNF production in MDM from these two donors (data not shown). When MDM from four of the donors (donors 1, 4, 9, and 12, whose monocytes were responsive to SP) were treated with SP (10^{-6} M) in the presence of LPS (0.1 ng/ml), synergistic effects were observed (Fig. 6). Experiments with the same donors after 2 to 4 months yielded results similar to those obtained in the previous experiments, and no significant individual variations in responses to SP at different times were observed (data not shown).

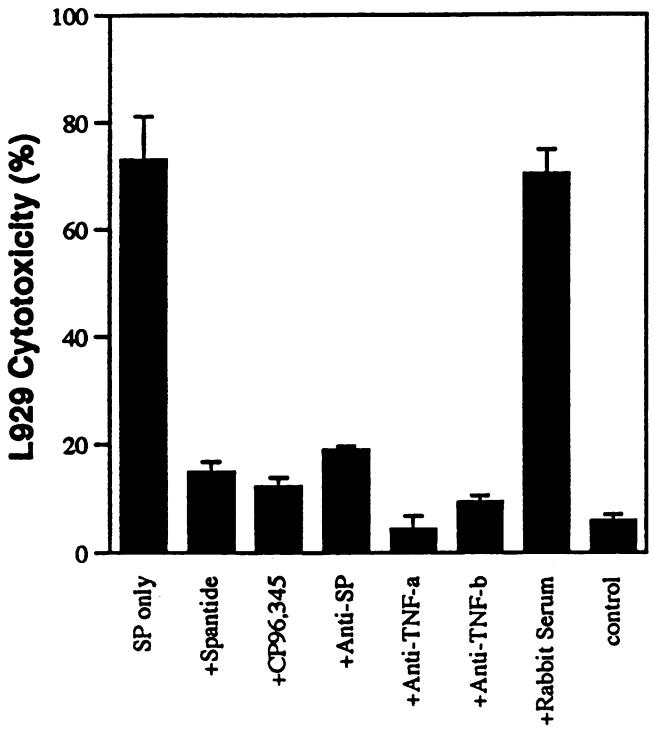

FIG. 4. Specificity of the neuropeptide effect. MDM cultured for 14 days were treated with SP at 10^{-6} M in the absence or presence of the SP antagonist spantide (10^{-5} M) or CP-96,345 (10^{-5} M), an anti-SP polyclonal antibody (1:200 dilution), or normal rabbit serum. To confirm the specificity of cytotoxic activity in cell supernatants, SP (10^{-6} M)-treated cell supernatants were incubated with anti-TNF-α or anti-TNF-β sera (1:200 dilution) or normal rabbit serum (1:200 dilution) for 1 h at 37°C before being added to L929 cell cultures. TNF production was determined with an L929 cytotoxicity assay. The results shown are means for triplicate cultures, representative of three experiments.

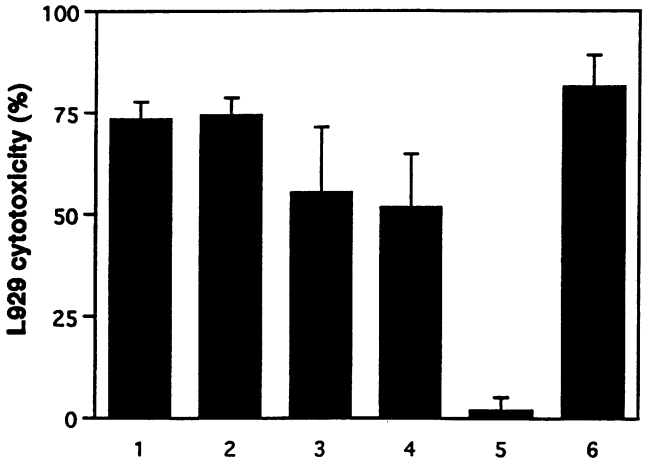

FIG. 3. Effect of SP, SP^{sar}, and SP fragments on TNF production. MDM cultured for 14 days were treated with SP (lane 1), SP^{sar} (lane 2), SP^{1-4} (lane 3), or SP^{5-11} (lane 4) at 10^{-6} M. After 24 h of incubation, supernatants were collected for a cytotoxicity assay with L929 cells. The negative control (lane 5) is supernatants collected from untreated MDM cultures. Recombinant TNF-α (0.1 ng/ml) (lane 6) was added to cultures of L929 cells as a positive control. The results shown are means for triplicate cultures, representative of two experiments.

DISCUSSION

Mononuclear phagocytes produce a wide array of potent mediators and cytokines which modulate the inflammatory response (5, 20). After a short period of maturation, monocytes leave the bone marrow, migrate to the bloodstream, in which they circulate for 1 to 3 days, and then migrate randomly to various organs and body cavities, in which they differentiate into macrophages (5, 20). Macrophages remain at these sites

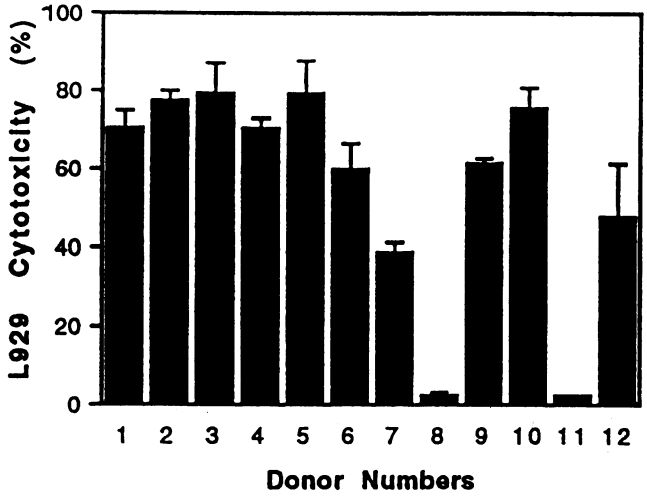

FIG. 5. Effects of SP on TNF production in MDM from 12 donors. MDM obtained from different donors (1 to 12) and cultured for 14 days were treated with SP at 10^{-6} M for 24 h. Supernatants were collected for a cytotoxicity assay with L929 cells. Untreated MDM culture supernatants from each donor were used as controls. Because less than 5% cytotoxicity was observed for the control supernatants from each of the 12 donor MDM cultures, such data are not included in this figure. The results shown are means for triplicate cultures.

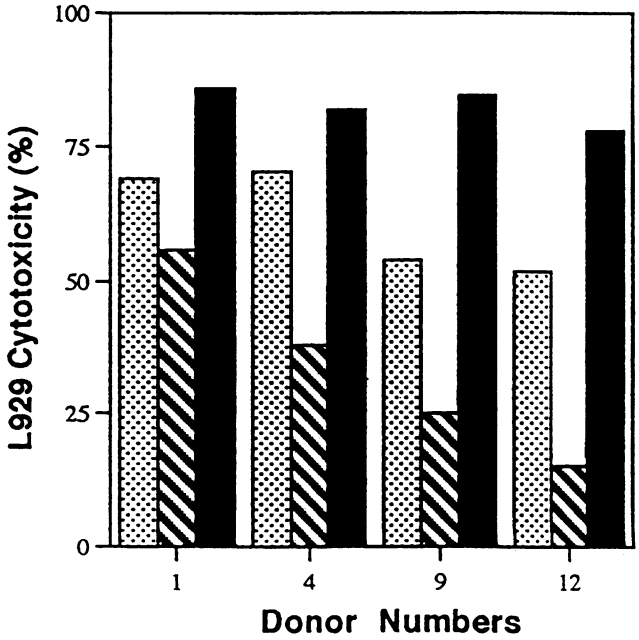

FIG. 6. TNF production by MDM treated with LPS and SP. MDM obtained from four donors (donors 1, 4, 9, and 12) and cultured for 14 days were treated with LPS (0.1 ng/ml) (▧) or SP (10^{-6} M) (▨) or both (■). Supernatants were harvested 24 h posttreatment and analyzed for TNF activity in L929 cells. Untreated MDM culture supernatants from each donor were used as controls.

for 1 to 3 months and then die, locally or in draining lymph nodes. The maturation process alone may be associated with changes in genetic regulatory events which lead to alterations in cellular function, such as cytokine expression (19). Activated alveolar macrophages and cultured MDM show increased production of TNF-α in comparison with freshly isolated monocytes (25). Therefore, it is very important to study functions of MDM under various conditions in vitro. The nervous system modulates immunological and inflammatory responses, most likely through neuropeptides such as SP (28). SP modulates neuroendocrine-immune system functions (22). Lotz et al. (16) have shown that SP and substance K induce the release of IL-1, TNF-α, and IL-6 in freshly isolated human monocytes (24 to 48 h in vitro). We examined the effects of SP on MDM. The experiments reported here demonstrate that TNF production in MDM cultured for 14 days is increased by SP in the presence or absence of LPS and in a dose-dependent manner. Our data indicate that both TNF-α and TNF-β were present in SP-stimulated MDM supernatants, since antibodies to both monokines neutralized the cytotoxic effects of the supernatants on L929 cells (Fig. 4). MDM from 10 of 12 donors showed TNF production induced by SP at 10^{-10} to 10^{-6} M. Although it is difficult to directly extrapolate the in vitro SP concentrations used in our experiments to in vivo microenvironments, concentrations of 10^{-10} to 10^{-8} M are likely achieved within the small intercellular space between the neuronal fibers in contact with tissue macrophages.

The mechanism of interaction between SP and monocytes/macrophages is not known; consequently, it is difficult to explain the variability of the response to SP of MDM from different donors. As described above, MDM from 2 of 12 donors showed no response to SP (Fig. 5). In addition, variability of the response to LPS of MDM from different donors was also observed. One possibility is that a heterogeneous population of monocytes is present in the cultures and the response to SP depends on the prevalence of certain cell subgroups and their different characteristics with regard to differentiation and endogenous ability to produce TNF (19, 20). A second possibility is the variability of SP receptor expression on MDM from different donors. SP receptors have been shown on human CD4-positive T cells, CD8-positive cells (23), murine lymphocytes (31), human IM-9 lymphocytes (22), murine B cells (25), and guinea pig macrophages (9). SP binding to human monocytes/macrophages was recently demonstrated (17). The SP antagonists spantide and CP-96,345, a potent nonpeptide antagonist of the SP (NK-1) receptor (30), blocked SP effects on TNF production in MDM, providing further indirect evidence for the receptor-mediated nature of the response. A third possibility is in vitro-induced loss of the SP receptor on MDM, although no such evidence has been reported. This assumption is based on the fact that fresh monocytes from any given donor are responsive to SP, as determined by monokine production (TNF, IL-1, and IL-6) (15, 16).

Bennett et al. showed that human peripheral monocytes can be cultured in a long-term serum-free system (2, 3). The suppressive effect of autologous serum on cultured human monocytes with regard to TNF production has been reported (29). Since endotoxin or undefined growth factors in serum may interfere with SP, we used serum- and protein-free medium for the experiments. No change in cell viability was observed, as determined by trypan blue exclusion and the presence of adherent cells.

In this study, we showed that SP alone enhances TNF release in human MDM, a result consistent with those of Lotz et al. (16) and Laurenzi et al. (15). Nevertheless, Laurenzi et al. found that monocytes from some subjects responded only to SP when LPS was present. Martin et al. reported that SP alone or in synergy with LPS did not stimulate TNF or IL-1 production in rat brain microglia (18). Our experiments

showed that SP alone or in a synergistic fashion with LPS stimulated TNF production in MDM from SP responders, suggesting that a different mechanism may be involved in SP-induced TNF production in MDM.

We have demonstrated that SP induces human MDM to produce TNF in the presence or absence of LPS. This study adds further support for the link between the neurotransmitter SP and macrophages. SP-macrophage interactions may exemplify the emerging intimate connections between neuropeptides and the immune system that have only recently begun to be elucidated.

ACKNOWLEDGMENTS

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H.-R.L. and W.-Z.H. contributed equally to this research.

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