Nuclear factor-κB activation in human monocytes stimulated with lipopolysaccharide is inhibited by fibroblast conditioned medium and exogenous PGE_2

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Received 18 November 1996

Abstract The nuclear factor κB (NF-κB) is thought to be crucially involved in the gene activation of several cytokines, including tumor necrosis factor α (TNF). Previously, we showed that fibroblast conditioned medium (FCM) is able to inhibit both TNF mRNA accumulation and protein release in peripheral blood-derived human monocytes (PBM) stimulated with lipopolysaccharide (LPS). In this study we have investigated the effect of FCM on the LPS-induced DNA-binding activity of NF-κB, by means of electrophoretic shift assay (EMSA). We provide evidence that FCM strongly inhibits the LPS-induced NFκB activation in PBM. Furthermore, we show that exogenous PGE_2 mimics the NFκB inhibitory effect of FCM. On the other hand, FCM produced in the presence of indomethacin does not inhibit NF-κB activation by LPS. Our results lend further support to the hypothesis that inflammatory and immune responses of monocytes/macrophages may be modulated at the molecular level by signals originating from tissue structural cells such as fibroblasts.

Key words: Nuclear factor κB; Monocyte; Macrophage; Lipopolysaccharide; Fibroblast; Prostaglandin E_2

1. Introduction

Nuclear factor κB (NF-κB) is a heterodimeric transcription factor composed of p50 and p65 subunits that belong to the protein family NF-κB\Rel\Dorsal (reviewed in [1–3]). All proteins of this family, which currently include the proto-oncogene Rel, NF-κB1 (p105 or p50), NF-κB2 (p100 or p52), Rel A (or p65), Rel B in mammalian cells and the *Drosophila* morphogen Dorsal (nomenclature in [4]), share an extensive sequence homology at their approx. 300-amino-acid N-terminal region containing both the dimerization and DNA-binding domains as well as the nuclear localization signal (NLS). NF-κB was originally identified as an inducible B cell-specific factor able to bind to the decameric κB motif in the intronic κ light chain enhancer [5]. NF-κB is now recognized to be quite ubiquitously present in most cell types in a cytoplasmic, inactive form. In fact, an inhibitor protein, IκB(s), typically sequesters the NF-κB complex in the cytoplasm by masking NLS and DNA-binding domains. IκB(s) also belong to a family of related proteins which include IκBα, IκBβ, IκBγ, Bcl-3 and *Drosophila* Cactus [6]. A large array of stimuli, including viruses, bacterial lipopolysaccharide (LPS), antigen-receptor engagement, stress factors and cytokines, may induce a rapid IκB dissociation/degradation and subsequently NFκB translocation to the nucleus where it binds decameric κB motifs of the consensus sequence 5′-GGGRNNYYCC-3′ (R = purines, N = any nucleotide, Y = pyrimidines) (references in [7]). There is a large set of genes with putative κB motifs in their *cis*-acting elements and in many of these the NF-κB binding appears crucial to gene regulation. These genes encode defense and signaling proteins including cell surface molecules involved in immune function such as immunoglobulin κ light chain, class I and II major histocompatibility complex (MHC) and cytokines such as Interleukin-1β (IL-1 β), IL-2, IL-6, IL-8, Interferon β (INF β), G-CSF, GM-CSF and TNF (reviewed in [8]). In the monocyte cell system, NFκB nuclear activity has been shown to be constitutive and thus responsible for the low, but detectable, level of TNF transcription [9]. The monocyte cell system (promonocytes, monocytes and tissue macrophages) represents a crucial element not only in immune defense, in that these cells exert phagocyte function, present processed antigen to T cells and orchestrate the immune response by cytokine secretion (extensive list of references in [10]), but also in the regulation of tissue turnover and repair, inflammation, atherosclerosis and tumor growth [11–13]. Previously we showed [14] that fibroblasts, the most ubiquitous tissue cells, are able to modulate LPS-induced TNF production in peripheral blood monocytes (PBM). In this study, we have investigated the effect of soluble factor(s) released by actively proliferating lung fibroblast on NFκB activation in adherent human PBM stimulated with LPS. We provide evidence that FCM strongly inhibits the LPS-induced nuclear mobilization of NFκB. Furthermore, we have investigated the effect of exogenous PGE_2 on NF-κB activation, as we had detected significant amounts of spontaneously released PGE_2 in FCM and observed that FCM lost its inhibitory capability when it was produced in the presence of the cyclooxygenase inhibitor indomethacin. We show that exogenous PGE_2 inhibits, in a dose-dependent manner, the NFκB nuclear mobilization induced by LPS in human adherent PBM.

2. Materials and methods

2.1. Fibroblast conditioned medium

Fibroblast conditioned medium was generated from cultures of 2×10^6 fibroblasts incubated for 24 h in 10 ml of supplemented RPMI (Gibco, Paisley, UK): 10% fetal calf serum (Gibco), penicillin/streptomicin, L-glutamine (2 mM). In some cases fibroblasts were cultured in the presence of indomethacin (5×10^{-5} M, Sigma, St. Louis, MO). All supernatants were then centrifuged and stored in aliquots at −20°C until use.

2.2. Fibroblast cultures

Primary lines of normal human adult lung fibroblasts were estab-

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PII S0014-5793(96)01409-3

lished using an outgrowth from explants from histologically normal areas of surgical lung specimens from patients undergoing resective surgery for cancer according to the method of Jordana and co-workers [15]. Aliquots of the cells were frozen and stored in liquid nitrogen. In all experiments we used cell lines at a passage earlier than the eighth.

2.3. Monocyte isolation procedure

Peripheral blood monocytes (PBM) were obtained from healthy donors as described by Colotta and associates [16] under LPS-free conditions. Monocyte purity was >90% as assessed by morphology and non-specific esterase staining.

2.4. Adherent monocyte cultures

PBM were plated at a concentration of 5×10^5 cells/ml of supplemented (as described above) RPMI in 10 cm dishes and allowed to adhere in a humidified atmosphere of 5% CO_2 at 37°C. After 2 h, the medium was removed and fresh supplemented RPMI or FCM was added. Cells were then immediately stimulated with lipopolysaccharide (LPS from *E. coli*, Sigma, 10 μg/ml) and incubated for 2 h.

2.5. Prostaglandin E_2 assay

Concentrations of PGE_2 in FCM were determined using a commercially available enzyme immunoassay, according to the manufacturer's instructions (PGE_2 enzyme immunoassay kit, Cayman Chemical Co., Ann Arbor, MI, USA). The assay is sensitive to 3.9 pg/ml of PGE_2. All samples were determined in triplicate.

2.6. Nuclear extract preparation

The nuclear protein extracts were prepared according to the protocol of Dignam et al. [17] with some modifications. Briefly, after the established times of culture, the cells were washed twice with PBS and then harvested with a rubber policeman in 1 ml cold buffer containing: 10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin. After 15 min on ice, Nonidet P40, 0.1% (v/v), was added to the homogenates and the tubes were vigorously rocked. Then the homogenates were centrifuged ($1\times10^4\times g$) for 50 s in a microcentrifuge at 4°C. The nuclear pellets were resuspended in 30 ml of ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and vigorously rocked at 4°C for 15 min. The nuclear extracts were centrifuged for 10 min at 4°C and the supernatants were stored in aliquots at −80°C. The protein content was estimated with a kit for protein microdetermination (Biorad).

2.7. Electrophoretic mobility shift assay (EMSA) and gel supershift

Nuclear extracts (5 μg of protein) were incubated with 30 000 cpm of a commercially obtained double-stranded oligonucleotide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), of 20 bp containing the (underlined) NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) labeled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase (Boehringer, Mannheim, Germany) and purified on a spin Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Incubations were performed for 30 min in the presence of 50 μg/ml of poly(dI-dC), 1 mg/ml nuclease-free BSA and binding buffer containing 100 mM Tris base, 400 mM KCl, 5 mM DTT, 0.5% NP-40, 25% glycerol. Samples were then electrophoresed through a 4% polyacrylamide gel for 2 h at 200 V. Gels were exposed to X-ray film with intensifying screens for 6–18 h. In some EMSAs unlabeled oligonucleotide was added in molar excess (100×) as specific binding competitor for 30 min at room temperature before incubating samples. For gel supershift nuclear extract were pre-incubated (20 min at room temperature) with anti p-50 or anti p65 antibodies (SantaCruz Biotechnology).

3. Results

3.1. Effect of fibroblast conditioned medium (FCM) on LPS-induced NFκB activation

Preliminary experiments, with very similar results, were performed by pre-incubating monocytes in FCM for 60, 30, 15 or 0 min before LPS treatment. Thus, in all experiments LPS was added simultaneously with FCM. As shown in Fig. 1, the specific DNA binding of NFκB complexes induced in adherent human PBM stimulated with LPS was strongly inhibited in the presence of FCM. The specificity of NFκB binding was shown by the complete disappearance of labeled complexes, which was indicated as I and II, in the competition assay with a molar excess of unlabeled probe and by a supershift of complexes in the presence of anti p-50 and/or anti p65 antibodies (data not shown). According to Ziegler-Heitbrock et al. [18], we assume that complex I represents the p50-p65 heterodimer (NFκB) and complex II represents the p50-p50 homodimer. It is noteworthy that in 3 out of 10 experiments the NF-κB complexes were fully activated in resting monocytes and no further activation was induced by LPS. In these experiments FCM reduced the baseline level of NFκB DNA-binding activity (data not shown).

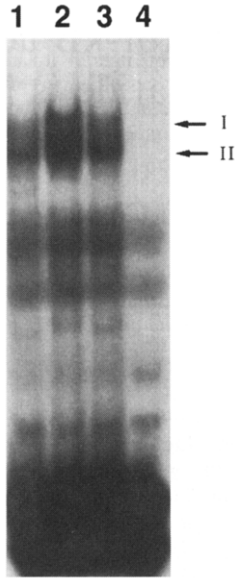

Fig. 1. Fibroblast conditioned medium (FCM) inhibits LPS-induced NF-κB activation in adherent human PBM. Electrophoretic mobility shift assay (EMSA) result (1 of 10 experiments) obtained using a ^{32}P-labeled oligonucleotide containing an NF-κB binding sequence and nuclear extracts from unstimulated monocytes (lane 1), monocytes stimulated with LPS (10 μg/ml) in the absence (lane 2) or presence of FCM (lane 3). As a specificity control, nuclear extracts as in lane 2 were pre-incubated with a 100× molar excess of cold probe (lane 4). Immunologically characterized NF-κB complexes are denoted I (p50-p65) and II (p50-p50).

3.2. PGE_2 amounts in FCM

In order to identify the soluble factor(s) responsible for blocking LPS-induced NFκB activation we measured the PGE_2 content in FCM. In fact, fibroblasts spontaneously released PGE_2 into the medium (1.208 ± 0.140 ng/ml, equivalent to about 3.4×10^{-9} M). We also produced FCM in the presence of indomethacin and determined PGE_2. As expected, the addition of indomethacin to fibroblast cultures caused a 100-fold reduction of PGE_2 content (19 ± 7 pg/ml equivalent to 5.67×10^{-12} M).

3.3. Effect of PGE_2-depleted FCM on LPS-induced NFκB activation

As Fig. 2 shows, the FCM generated in the presence of indomethacin hardly reduced the NFκB activity.

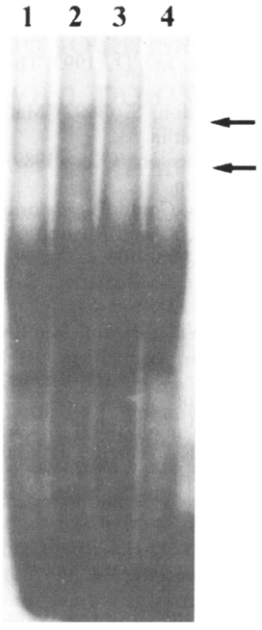

Fig. 2. PGE_2-depleted FCM does not inhibit LPS-induced NF-κB nuclear mobilization. EMSA result (1 of 3 experiments) obtained using a ^{32}P-labeled oligonucleotide containing an NF-κB binding sequence and nuclear extracts from unstimulated monocytes (lane 1), monocytes stimulated with LPS, 10 μg/ml (lane 2), monocytes stimulated with LPS in the presence of FCM generated with indomethacin (lane 3) and monocytes stimulated with LPS in the presence of normal FCM (lane 4). Activated NF-κB complexes are indicated.

3.4. *Effect of exogenous PGE_2 on LPS-induced NFκB activation*

In our experiments, the addition of exogenous PGE_2, at the same time as LPS stimulation, mimicked the FCM inhibitory effect on NFκB activation by LPS. Fig. 3 demonstrates that the inhibitory effect started from a concentration threshold of PGE_2 (10^{-9} M) which is comparable to the PGE_2 concentration in FCM.

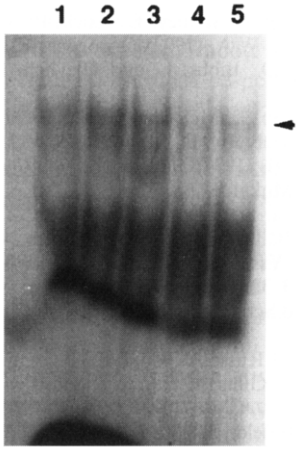

Fig. 3. Dose-dependent effect of exogenous PGE_2 on LPS-induced NF-κB nuclear mobilization. EMSA result (1 of 3 experiments) obtained using a ^{32}P-labeled oligonucleotide containing an NF-κB binding sequence and nuclear extracts from unstimulated monocytes (lane 1), monocytes stimulated with LPS, 10 μg/ml (lane 2) and monocytes stimulated with LPS in the presence of increasing concentrations of exogenous PGE_2 (0.1, 1, 10 ng/ml: lanes 3, 4, 5, respectively). The arrow indicates the unresolved NFkB complexes.

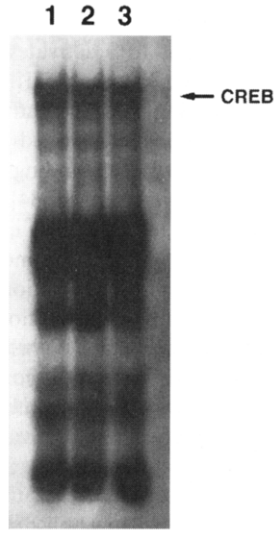

Fig. 4. FCM does not influence the activity of the cyclic AMP response element binding (CREB) transcription factor. EMSA result using a ^{32}P-labeled oligonucleotide containing a CREB binding sequence and nuclear extracts from unstimulated monocytes (lane 1), monocytes stimulated with LPS, 10 μg/ml (lane 2) and monocytes stimulated with LPS in the presence of FCM (lane 3). Two adjacent CREB complexes [19] are indicated.

3.5. *Effect of FCM on the activity of the unrelated transcription factor CREB*

To verify the specificity of the FCM inhibition of LPS-induced NFκB activity we also investigated the possible effect on the activity of the cyclic AMP response element binding (CREB) transcription factor. As reported by others [19], nuclear extracts from monocytes (stimulated or not with LPS) showed a strong binding activity with labeled CRE oligonucleotides in EMSA. As shown by Fig. 4, FCM treatment did not affect CREB binding activity in monocytes.

4. Discussion

Mononuclear phagocytes are important regulators of immune, inflammatory and fibrotic response. These functions are mediated through the production of several cytokines, including TNF, IL-1, IL-6, IL-8 and GM-CSF. In response to pathological stimuli, blood monocytes extravasate to the tissue and orchestrate the immune/inflammatory response by secretion of such cytokines. This process is normally tuned by a fine balance of stimulatory and inhibitory signals. A failure of this balanced tuning may lead to pathological conditions such as septic shock or chronic inflammation. We previously reported that tissue structural cells such as fibroblasts may modulate immune/inflammatory responses [14,20–23]. In this report, we show that a soluble factor(s) spontaneously released by actively proliferating lung fibroblasts is able to inhibit specifically the nuclear mobilization of the pivotal transcription factor NFκB in human adherent PBM stimulated with LPS. Mechanisms by which LPS activates mononuclear phagocytes, its major cellular target, have not yet been completely characterized. LPS provokes multiple effects on mononuclear phagocytes including NF-κB mobilization [24], cytokine gene activation and release [25–27]. Several cell surface molecules such as CD14, CD11/CD18 are able to recognize the lipid A portion of the polysaccharide region of the LPS molecule [28,29]. The interaction of LPS with cell membrane

receptors may trigger different signal transduction pathways. There is much evidence that LPS activates protein kinase C (PKC) [30], protein tyrosine kinases (PTK [31,32]), mitogen-activated protein (MAP) kinases [33] and a pair of closely related MAP kinase homologues, termed CSBPs (cytokine-suppressive anti-inflammatory drug binding proteins), recently cloned and biochemically characterized [34]. In any case, protein phosphorylation seems to be the key event in the mononuclear phagocyte response to LPS [35], inducing NF-κB nuclear mobilization. In fact, phosphorylation has been shown to be a fundamental step in the activation of NF-κB [36]. Even though we can only speculate on which LPS-triggered pathway(s) leading to NFκB activation could be affected by FCM, the inhibitory effect of FCM soluble factor(s) could rely on the impairment of kinase activation triggered by LPS. Nevertheless, in order to identify the inhibitory factor(s) we measured PGE_2 content (10^{-9} M) in the FCM, as it has been shown that PGE_2 is able to abolish the NFκB binding activity in stimulated human T_h cell clones [37] as well as in mesangial cells [38]. Moreover, it has been shown that PGE_2 can act as a negative regulator of various lymphocyte and macrophage activities including cytotoxicity for tumor target cells [39], response to migration inhibitors [40], phorbol myristate acetate (PMA)-triggered production of ROIs [41] and cytokine production [42,43]. In this report, we show that exogenous PGE_2 (10^{-9}–10^{-8} M) is able to suppress LPS-induced NFκB activation in human adherent PBM. We also show that FCM produced in the presence of indomethacin, a cyclooxygenase inhibitor which efficaciously depleted PGE_2, failed to inhibit, at least in part, NFκB activation. These data indicate a major role of PGE_2 in the inhibition exerted by FCM on LPS-induced NFκB activation, even though we cannot rule out the contribution of other soluble factor(s). Based on our in vitro findings, we hypothesize that the exchange of cross-talks between monocytes/macrophages and fibroblasts, which relies on soluble factors such as cytokines and PGE_2, could be an important in vivo mechanism leading to the modulation of inflammatory/immune response, as well as fibrotic response, via the control of the pivotal transcription factor NFκB.

Acknowledgements: This work was supported by the 'Ministero dell'Università e della Ricerca Scientifica (MURST) 40%', 'Consiglio Nazionale delle Ricerche (CNR)', and 'Associazione Italiana Ricerca sul Cancro (AIRC)'. The authors are indebted to Prof. B Cassia and the staff of the transfusional centre of the 'Garibaldi' Hospital, Catania, for kindly supplying buffy coats.

References

[1] Siebenlist, U., Franzoso, G. and Brown, K. (1994) Annu. Rev. Cell. Biol. 10, 405–455.
[2] Grilli, M., Jason, J.-S. and Lenardo, M.J. (1993) Syst. Int. Rev. Cytol. 143, 1–62.
[3] Liou, H.-C. and Baltimore, D. (1993) Curr. Opin.. Cell Biol. 5, 477–487.
[4] Nabel G.J. and Verma, I. (1993) Genes Devel. 7, 2063.
[5] Sen, R. and Baltimore, D. (1986) Cell 46, 705–716.
[6] Gilmore, T.D. and Morin, P.J. (1993) Trends Genet. 9, 427–433.
[7] Bauerle, P.A. (1991) Biochim. Biophys. Acta 1072, 63–80.
[8] Bauerle, P.A. and Henkel T. (1994) Annu. Rev. Immunol. 12, 141–179.
[9] Frankenberger, M., Pforte, A., Sternsdorf, T., Passlick, B., Baeuerle, P.A. and Ziegler-Hetbrock, H.W. (1994) Biochem. J. 304, 87–94.
[10] Seljelid, R. and Eskeland T. (1993) Eur. J. Haematol. 51, 267–275.
[11] Lewis, C.E. and McGee, J.O.'D. (1992) The Macrophage, Oxford University Press, Oxford.
[12] Russel, S.W. and Gordon, S. (1992) Macrophage Biology and Activation, Springer, Berlin.
[13] Zembala, M. and Asherson, G.L. (1989) Human Monocytes, Academic Press, London.
[14] Vancheri, C., Crimi, N., Conte, E., Pistorio M.P., Mastruzzo C., Lamicela, M., Messina, A. and Mistretta, A. (1996) Am. J. Respir. Cell Mol. Biol. 14, 460–466.
[15] Jordana, M., Schulman, J., McSharry, C. and Gauldie, J. (1988) Am. Rev. Respir. Dis. 137, 579–584.
[16] Colotta, F., Peri, G., Villa, A. and Mantovani, A. (1984) J. Immunol. 132, 936–944.
[17] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[18] Ziegler-Heitbrock, H.W., Sterndorf, T., Liese, B.B., Weber C., Wedel, A., Schreck, R., Bauerle, P. and Strobel, M. (1993) J. Immunol. 151, 6986–6993.
[19] Newell, C.L., Deisseroth, A.B. and Berestein, G.L. (1994) J. Leuk. Biol. 56, 27–35.
[20] Ohtoshi, T., Vancheri, C., Cox, G., Gauldie, J., Dolovich, J., Denburg, J.A. and Jordana, M. (1991) Am. J. Respir. Cell Mol. Biol. 4, 255–263.
[21] Vancheri, C., Ohtoshi, T., Cox, G., Xaubet, A., Abrams, J.S., Gauldie, J., Dolovich, J., Denburg, J. and Jordana, M. (1991) Am. J. Respir. Cell Mol. Biol. 4, 11–17.
[22] Vancheri, C., Gauldie, J., Bienenstock, J., Cox, G., Schicchitano, R., Stanisz, A. and Jordana, M. (1989) Am. J. Respir. Cell Mol. Biol. 1, 289–295.
[23] Cox, G., Ohtoshi, T., Vancheri, C., Denburg, J.A., Dolovich, J., Gauldie, J. and Jordana, M. (1991) Am. J. Respir. Cell Mol. Biol. 4, 525–531.
[24] Mueller, J.M., Ziegler-Hetbrock, H.W. and Baeuerle, P.A. (1993) Immunobiology 187, 233–256.
[25] Cavaillon, J.M. and Haefner-Cavaillon, N. (1990) Cytokine 2, 313.
[26] Trinchieri, G. (1991) Immunol. Res. 10, 89.
[27] Liebermann, T.A. and Baltimore, D. (1990) Mol. Cell. Biol. 10, 2327.
[28] Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J.and Mathison J.C.(1990) Science 249, 1431.
[29] Bright, S.W., Chen, T.Y., Flebbe, L.M., Lei, M.G. and Morrison, D.C. (1990) J. Immunol. 145, 1.
[30] Wightman, P.D. and Raetz, C.R.H. (1984) J. Biol. Chem, 259, 10048.
[31] Weinstein, S.L., Sanghera, J.S., Lemke, K., DeFranco, A.L. and Pelech, S.L. (1992) J. Biol. Chem. 267, 14955.
[32] Boulet, I., Ralph, S., Stanley, E., Lock, P., Dunn, A.R., Green, S.P. and Phillips, W.A. (1992) Oncogenes 7, 703.
[33] Han, J., Lee, J.D., Bibbs, L. and Ulevitch, R.J. (1994) Science 265, 808–811.
[34] Lee, J.C., Laydon, J., McDonnel, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W. et al. (1994) Nature 372, 739–746.
[35] Ishikawa, Y., Mukaida, N., Kuno, K., Rice, N., Okamoto, S.I. and Matsushima, K. (1994) J. Biol. Chem. 270, 4158–4164.
[36] Ghosh, S.and Baltimore, D. (1990) Nature 344, 678–682.
[37] Watanabe, S., Yssel, H., Harada, Y. and Arai, K. (1994) Int. Immunol. 6, 23–32.
[38] Satriano, J. and Scholondorff, D. (1994) J. Clin. Invest. 94, 1629–1633
[39] Taffet, S.M. and Russel, S.W. (1981) J. Immunol. 126, 424–432.
[40] Koopman, W.J., Gillis, M.H. and David, J.R (1973) J. Immunol. 110, 1609–1614.
[41] Metzger, Z., Hoffeld., J.T. and Oppenheim, J.J. (1981) J. Immunol. 127, 109–114.
[42] Kunkel, S.L., Chensue, S.W. and Phan, S.H. (1986) J. Immunol. 136, 186–192.
[43] Kunkel, S.L., Spengler, M., May, M.A., Spengler, R., Larrick, J. and Remick, D. (1988) J. Biol. Chem. 263, 5380–5384.