RECOMBINANT TNF-α MEDIATED REGULATION OF THE IκB-α/NF-κB SIGNALING PATHWAY: EVIDENCE FOR THE ENHANCEMENT OF PRO- AND ANTI-INFLAMMATORY CYTOKINES IN ALVEOLAR EPITHELIAL CELLS

John J. Haddad

The signaling transduction mechanism mediated by tumor necrosis factor-α (TNF-α) in the alveolar epithelium is not well characterized. It was subsequently hypothesized that recombinant murine TNF-α (rmTNF-α) selectively regulates the inhibitory κB (IκB-α)/nuclear factor-κB (NF-κB) pathway and interferes with the endogenous biosynthesis of pro-inflammatory (stimulatory) and anti-inflammatory (inhibitory) cytokines. The cytokine rmTNF-α induced, in a time- and dose-dependent manner, the degradation of IκB-α within the cytosolic compartment, an effect associated with up-regulating its phosphorylation. This allowed the biphasic regulation of selective NF-κB subunit nuclear translocation, thereby mediating a dual excitatory mechanism on NF-κB activation. The immunoregulatory effect of rmTNF-α was associated with a time-dependent induction of pro-inflammatory [interleukin (IL)-1β, IL-6 and TNF-α] and anti-inflammatory (IL-10) cytokine biosynthesis. These results indicate a novel involvement of an IκB-α/NF-κB-sensitive pathway mediating the effect of TNF-α, which is associated with an autocrine, endogenous mechanism mediating the regulation of cytokine signaling.

There is increasing evidence implicating the alveolar epithelium as a dynamic barrier that plays an important role in regulating the inflammatory and metabolic responses to oxidative stress and the accompanying inflammatory signal.6–9 Sepsis, endotoxemia, and other critical illnesses in the lung.6–9 The respiratory epithelium is a primary target of an inflammatory/infectious condition at the epithelial-blood interface and is itself capable of amplifying an inflammatory signal by recruiting inflammatory cells and by producing inflammatory mediators.1–5 Many of the side effects of lipopolysaccharide-endotoxin (LPS), derived form the cell wall of gram negative bacteria, and other inflammatory mediators such as tumor necrosis factor-α (TNF-α), are secondary to the overproduction of pro-inflammatory mediators, such as the pleiotropic cytokines, which exacerbate the pathophysiological condition by activating and recruiting inflammatory cells.6–9 Therefore, the suppression of a pro-inflammatory signal, and the down-stream conjugated inflammatory pathways, and augmentation of a counter-inflammatory response has been a major focus of the approach to the treatment of inflammatory diseases. For instance, glucocorticoids,10 extracellular purines,11 phosphodiesterase selective inhibitors,12,13 pyrimidylpiperazine,14–16 and adrenoreceptor agonism/antagonism17 have been widely used to counteract the effects of inflammatory cytokines and subsequently suppress the protracted pathophysiological conditions in vitro and in vivo.

Although the transcription factor NF-κB has been originally recognized in regulating gene expression in B-cell lymphocytes,18 subsequent studies have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of pro-inflammatory cytokines.19–21 The translocation and activation of NF-κB in response to various stimuli are sequentially organized at the molecular level.18,21 In its inactive state, the heterodimeric NF-κB, which is mainly composed of two subunits, p50 (NF-κB1) and p65 (RelA), is present in the cytoplasm associated with its inhibitory protein, IκB1,18,21,22
Upon stimulation, such as with cytokines and LPS, IkB-α, the major cytosolic inhibitor of NF-κB, undergoes phosphorylation on serine/threonine residues, ubiquitination and subsequent proteolytic degradation, thus unmasking the nuclear localization signal on p65 subunit and allowing nuclear translocation of the complex. This sequential propagation of signaling ultimately results in the release of NF-κB subunits from IkB-α, thereby promoting translocation and initiation of gene transcription and regulation.23,24

TNF-α is a potent inflammatory cytokine, which exerts its pleiotropic activities through ligand-induced cross-linking of specific receptors, virtually present in almost all cell types.23 During inflammation, TNF-α released in the inflammatory environment transiently activates immune and non-immune cells, thereby causing a rapid but transient release of reactive oxygen species (ROS), a crucial part of the defence mechanism against invading microbial pathogens and tumor cell metastasis.23 The signaling mechanism mediating the effect of TNF-α in the respiratory epithelium, however, has not been well characterized and has yet to be ascertained. The present investigation has attempted, therefore, to unravel the role that recombinant TNF-α plays in regulating the IkB-α/NF-κB signaling transduction pathway and the downstream cytokine transduction. In particular, TNF-α mediated regulation of NF-κB selective subunit translocation and subsequent activation was shown to be dependent on IkB-α phosphorylation and degradation within the cytosolic compartment. This regulatory effect was associated with the induction of pro-inflammatory (IL-1β, IL-6 and TNF-α) and anti-inflammatory (IL-10) endogenous cytokine biosynthesis, indicating the involvement of an autocrine, cytokine-sensitive mechanism mediating the effect of TNF-α on IkB-α/NF-κB signaling.

RESULTS

*rmTNF-α mediated regulation of IkB-α signaling: Abundance versus phosphorylation state*

The modulatory role of rmTNF-α in regulating IkB-α phosphorylation and degradation is not well characterized in the alveolar epithelium. Subsequently, we designed a series of experiments to investigate this potential immunoregulatory role. The effect of rmTNF-α on IkB-α regulation is shown in Figure 1. A time-response analysis of the effect of rmTNF-α on IkB-α abundance and phosphorylation within the cytosolic compartment is shown in Figure 1A. The effect of rmTNF-α on IkB-α abundance was immediate, evident as early as 2 min (1/30 h) post addition of rmTNF-α, and continued to degrade in a time-dependent manner with variable abundance between 1 h and 48 h of stimulation (Fig. 1A). This inhibitory effect of rmTNF-α on IkB-α abundance was associated with up-regulating its phosphorylation in a biphasic, time-dependent manner (Fig. 1A). To assess for a relationship between the abundance of IkB-α and its phosphorylation state, we determined the efficient ratio R=[IkB-α]/[pIkB-α], as shown in Figure 1B. The histogram analysis revealed a biphasic effect of rmTNF-α on IkB-α (Fig. 1B). The protein expression of IkB-α within the cytosolic compartment decreased with rmTNF-α immediately after addition of the cytokine, in a time-dependent manner. The level of IkB-α expression and the amount of its phosphorylation were similar at 10 min (1/6 h); however, the level of phosphorylation exceeded its abundance, especially between 15 and 20 min (1/4–1/3 h) (Fig. 1B). Despite the observation that the abundance of IkB-α was relatively higher than its phosphorylation state between 30 min and 2 h, IkB-α abundance in comparison to the time period between 0 and 5 min is evidently lower, indicating an active phosphorylation state (Fig. 1B). Again, the level of IkB-α expression and the amount of its phosphorylation were similarly comparable at 4h–8 h, thereby its phosphorylation takes over at between 16 h and 24 h, thereafter exhibiting comparable ratio at 48 h (Fig. 1B). The dose-response curve at 30 min for the effect of rmTNF-α on the abundance of IkB-α is shown in Figure 1C. At a concentration of 0.1 ng/ml, rmTNF-α induced degradation of IkB-α, which continued to degrade in the cytosolic compartment with ascending rmTNF-α concentration (Fig. 1C). The housekeeping gene protein product, β-actin, was used as an internal reference for semi-quantitative loading per parallel lanes, as shown in Figure 1C.

The effect of rmTNF-α on the nuclear translocation of selective NF-κB subunits

As shown in Figure 2, rmTNF-α (10 ng/ml) mediated, in a time-dependent manner, the nuclear translocation of selective NF-κB subunits. Gel analysis of the abundance of these subunits within the nuclear compartment revealed a biphasic stimulatory effect (Fig. 2). rmTNF-α induced NF-κB1 (p50) accumulation in as early as 2 min post addition, then subsiding to re-elevate again after 30 min, when it remained abundant until 16 h, thereafter declining (Fig. 2). The effect of rmTNF-α on NF-κB2 (p52) is marginal during early exposure, but slightly increased after 0.5–1 h to become significantly abundant at around 16 h, thereafter remaining mildly in the nuclei (Fig. 2). In a manner roughly similar to the effect on NF-κB1, rmTNF-α allowed nuclear accumulation of RelA (p65) immediately in as early as 2 min post addition, declining thereafter, but re-elevating at 30 min and continuing to be strongly abundant in the nucleus afterwards (Fig. 2). The abundance of RelB (p68) was variable to the effect of rmTNF-α in comparison with either
abundance of p50 or p65 subunit. In the absence of rmTNF-α we observed constitutive abundance of p68 within the nucleus, variably affected, but not enhanced, by the addition of rmTNF-α (Fig. 2). The abundance of p68 roughly stood there at different time points, with particular availability at 20 min and at between 2 h and 24 h, as shown in Figure 2. Finally, the effect of rmTNF-α on c-Rel (p75) nuclear abundance (Fig. 2) revealed an immediate induction in as early as 2 min, thereafter subsiding to re-escalate at 30 min, subsiding again and re-appearing at 4–16 h. The housekeeping gene protein product, β-actin, was used as an internal reference for semi-quantitative loading per parallel lanes, as shown in Figure 2.

The effect of rmTNF-α on the nuclear activation of NF-κB: Biphasic DNA-binding analysis

In association with the differential, biphasic effect of rmTNF-α on NF-κB selective subunit nuclear translocation, there was a dose-dependent up-regulation of the DNA-binding activity of this transcription factor, as shown in Figure 3A. The activation of NF-κB increased in the presence of ascending concentrations of rmTNF-α, with effective maximal activation at 10 ng/ml (Fig. 3A). Histogram analysis of the gel-shifted bands revealed the 10 ng/ml concentration as the most effective and optimum concentration that induced maximum activation (Fig. 3B). The time-response analysis of the effect of rmTNF-α on NF-κB activation is shown in Figure 4A. In association with a dual regulation of the nuclear translocation of selective NF-κB subunits, rmTNF-α up-regulated the activation of this transcription factor in a biphasic manner (Fig. 4A). The activation in response to rmTNF-α was immediate in as early as 2–5 min post addition, thereafter subsiding, but remained active between 10 and 20 min. Maximal activation with rmTNF-α was observed at 30 min post addition and remained thereafter steadily active until 24 h culturing in the continuous presence of rmTNF-α, to become inactive beyond
this time point at 48 h (Fig. 4A). Histogram analysis of the gel-shifted bands exhibiting the time-response curve is displayed in Figure 4B.

Analysis of the effect of rmTNF-α on the pro- and anti-inflammatory cytokine biosynthesis

The novel interference of rmTNF-α in regulating 1xB-α/NF-κB signaling pathway was associated with a dual effect on extracellularly secreted cytokines. As shown in Figure 5A, rmTNF-α (10 ng/ml) up-regulated the production of IL-1β, in a time-dependent manner. There was an initial increase in IL-1β (~2–4 h) after addition of rmTNF-α. This was followed by an intermediate decrease (~8–16 h) and a maximum increase at about 24–48 h (Fig. 5A). Similarly, rmTNF-α induced the biosynthesis of IL-6 in a time-dependent manner, with maximum enhancement at around 24–48 h, as shown in Figure 5B. Of interest, the observation reported that rmTNF-α induced the endogenous production of TNF-α, effective at 16 h, which continued to increase at 24 h and maximize at 48 h, as shown in Figure 5C. The most intriguing observation of all is the ability of rmTNF-α to augment the secretion of a potent anti-inflammatory cytokine (IL-10) in a manner similar to its ability to up-regulate the production of pro-inflammatory cytokines. The concentration of IL-10 in the supernatant appeared at 8 h post addition of rmTNF-α, continued to increase exponentially to maximize at around 48 h (Fig. 5D).

DISCUSSION

To accommodate an ever changing microenvironment, cells adjust the pattern of gene expression by adaptive regulation of a host of transcription factors, which bind their respective cognate sites in the regulatory elements of targeted genes.21,24–26 NF-κB comprises the Rel family of inducible transcription factors that are key mediators in regulating the progression of the inflammatory process.20 Therefore, activation/regulation of the NF-κB/Rel transcription family, via nuclear translocation of cytoplasmic entities and complexes, plays a central role in the evolution of inflammation through regulation of genes essentially involved
in encoding pro-inflammatory cytokines and other inflammatory mediators. The NF-κB/Rel family includes five members: NF-κB1 [p50/p105 (p50 precursor)], NF-κB2 [p52/p100 (p52 precursor)], RelA (p65), RelB (p68) and c-Rel (p75). Despite the ability of most Rel members (with the exception of p68) to homodimerize, as well as form heterodimers, with each other, the most prevalent activated form of NF-κB is the heterodimer p50–p65, which possesses the transactivity domains necessary for gene regulation. The NF-κB members contain a Rel homology domain (RHD), which is responsible for dimer formation, nuclear translocation, sequence-specific consensus DNA recognition and interaction with inhibitory-κB (IκB), the cytosolic inhibitors of NF-κB. In unstimulated cells, NF-κB resides in the cytoplasm as an inactive NF-κB/IκB complex, a mechanism which hinders the recognition of the nuclear localization signal (NLS) by the nuclear import machinery, thereby retaining the NF-κB complex within the cytosol.

Although the inflammatory signals mediated by TNF-α are well recognized in other systems and cell models, this role in the fetal alveolar epithelium is not well characterized. The cytokine rmTNF-α induced, in a time- and dose-dependent manner, the degradation of IκB-α within the cytosolic compartment, an effect associated with up-regulating its phosphorylation. This requirement allowed the biphasic regulation of selective NF-κB subunit nuclear translocation (supershift analysis revealed the involvement of the p50–p65 complex; Data not shown), thereby mediating a dual excitatory mechanism on NF-κB activation. The immunoregulatory effect of rmTNF-α was also associated with the induction of pro-inflammatory (IL-1β, IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokine biosynthesis, indicating a novel involvement of an IκB-α/NF-κB-sensitive pathway mediating the effect of TNF-α in association with an autocrine, endogenous mechanism governing the regulation of cytokine signaling. In particular, the interference of this cytokine in regulating the phosphorylation of IκB-α, the major cytosolic inhibitor of NF-κB, suggested the involvement of an upstream kinase. In this respect, signals emanating from membrane receptors, such as those for IL-1 and TNF-α, activate members of the MEKK-related family, including NF-κB inducing kinase (NIK) and MEKK1, both of which are involved in the activation of IκB kinases, IKK1 and IKK2.
components of the IKK signalsome. 27,28 IKK1 and IKK2 were identified as components of the high-molecular weight complex containing a number of proteins involved in NF-κB regulation. 27,28 These kinases phosphorylate members of the IκB family, including IκB-α, at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the 26S proteasome. Therefore, the immunoregulatory potential of rmTNF-α in up-regulating the phosphorylation of IκB-α and its subsequent degradation strongly implicated an upstream kinase, probably the IKK complex, as a potential target for the inflammatory action of this cytokine. Moreover, the selective interference mediated by rmTNF-α in regulating the translocation/activation of NF-κB and the expression of its inhibitor (IκB-α) is of particular interest since it suggests that this compound’s regulatory role in the alveolar space resides within and/or above the upstream pathway regulating the phosphorylation of IκB-α, thereby modulating the downstream pathway governing NF-κB translocation and activation, and subsequently interfering with the regulation of cytokine signaling.

The promoters of genes encoding cytokines, particularly IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-8, IL-12 and TNF-α, 24–26 contain multiple cis-acting motifs including those that bind such transcription factors as NF-κB. Furthermore, the release of free NF-κB upon extracellular stimulation (TNF-α, for example) due to IκB phosphorylation and degradation, leads to DNA binding to initiate transcription of related genes, including immunoreceptors, cytokines and, interestingly, its own inhibitor, IκB-α. 24–28 Two unique features of the NF-κB/IκB complex system are deduced from its feedback regulation. The transcriptional activation of NF-κB triggers the synthesis of IκB, and NF-κB activated transcription is maintained by continuous degradation of IκB, which is sustained by an extracellular stimulus (in this particular case rmTNF-α). 25–28 Thus, the expression of IκB parallels both NF-κB activity and the duration of the activating extracellular stimulation, suggesting that this temporal parallelism between IκB accumulation/degradation and an effective external stimulation is a mechanism allowing dual, biphasic, regulation of NF-κB within the respiratory epithelium. Of particular interest, unraveling the downstream cytokine pathway that is regulated by NF-κB/IκB remains a major target for the search for novel therapeutic agents that tend to suppress the inflammatory signal mediated, but have the potential though to up-regulate a counter-inflammatory response. One such novel approach is the discovery

Figure 4. The time-response analysis of the effect of rmTNF-α on NF-κB.

(A) rmTNF-α up-regulated the activation of this transcription factor in a biphasic manner. The activation was immediate in as early as 2–5 min, thereafter subsiding, but remained active between 10 and 20 min, with maximal activation at 30 min, and continued to be steadily active until 24 h, to become inactive beyond this time point at 48 h. (B) Histogram analysis of the gel-shifted bands exhibiting the time-response curve. *P<0.05, **P<0.01, ***P<0.001, as compared with control (rmTNF-α=0 ng/ml). n=3 independent experiments performed with separate cell cultures.
reported in this investigation of the potential immunoregulatory role that rmTNF-α exhibits in augmenting an inflammatory signal, yet up-regulate an anti-inflammatory loop; however, whether a direct association between either pathway is established that is likely to regulate IκB-α/NF-κB signaling transduction has yet to be ascertained as part of the mechanism mediating the effect of TNF-α.

The so-called ‘pro-inflammatory’ cytokines, which include TNF-α, are involved in a plethora of cellular actions, particularly inflammatory/infectious conditions.29 Locally, these cytokines stimulate leukocyte proliferation, cytotoxicity, synthesis and release of proteolytic enzymes, and synthesis of prostaglandins that initiate a cascade of ‘secondary’ cytokine transcription/biosynthesis, including the amplification of TNF-α and other inflammatory mediators.29 These cytokines, for instance, have the ability to raise the thermoregulatory set point systemically and, via differential influences on the expression of iron binding proteins, mediate a redistribution of iron from extracellular to intracellular sites and stores. Moreover, inflammatory mediators orchestrate a metabolic reaction that reduces any energy consumption not directed at repelling the microbial pathogen, redirects host resources to the defence effort, and sets up a defence network that protects non-leukocyte cells from collateral damage by anti-microbial effectors.29 One such secondary cytokine, IL-10, is often referred to as a counter-inflammatory (inhibitory) cytokine because of its temporal association with the aforementioned processes, thereby acting to keep potentially destructive inflammatory responses from overshooting.29 The ability of rmTNF-α to mediate the biosynthesis of IL-10, a potent anti-inflammatory cytokine, remains of particular interest. IL-10 was originally identified as a cytokine inhibitory factor, which suppressed the biosynthesis of an array of pro-inflammatory mediators, including IL-1β, IL-4, IL-8, TNF-α, TNF-α, interferon-γ (IFN-γ), and granulocyte/macrophage colony stimulating factor (GMCSF).30 The level of regulation by IL-10 is both transcriptional and translational, raising the possibility of IL-10 acting on transcription factors involved in regulating inflammatory genes.30 It has also been reported that IL-10 selectively regulated NF-κB activation, a phenomenon well correlated with a dose-dependent inhibition of the release of pro-inflammatory cytokines.30 Moreover, we have previously shown that rhIL-10 down-regulated NF-κB activation, an effect associated with the inhibition of inflammatory cytokine biosynthesis.16 On the mechanism of action of IL-10, it seems that it involves the blockade of a reaction required for the release of IκB from the complex in intact cells.16,31 For example, certain experiments using cell-free preparations have suggested that certain protein kinases phosphorylate
IκB causing its release and allowing activation of the NF-κB complex. Despite the unequivocal observation therein reported which gave an evidence for the amplification of an anti-inflammatory pathway alongside the IκB-α/NF-κB/inflammatory cytokine pathway mediated by rmTNF-α, the molecular basis for this biphasic mechanism remains to be elucidated. However, the possibility of TNF-α mediating an endogenous regulatory pathway via IL-10 which requires a dual regulation of IκB-α/NF-κB signaling is likely to imply a novel feedback and/or feedforward mechanism working within the respiratory epithelium.

In summary, the results of the present investigation could be highlighted as follows: (i) The cytokine rmTNF-α induced, in a time- and dose-dependent manner, the cytosolic degradation of IκB-α, an effect associated with up-regulating its phosphorylation; (ii) this allowed the biphasic translocation of selective NF-κB subunits onto the nuclear compartment, thereby mediating a dual excitatory mechanism on NF-κB activation; and (iii) the immunoregulatory effect of rmTNF-α was associated with the induction of pro-inflammatory (IL-1β, IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokine biosynthesis. These results indicated the involvement of an IκB-α/NF-κB-sensitive pathway, which is associated with an autocrine, endogenous mechanism mediating the regulation of cytokine signaling.

MATERIAL AND METHODS

Chemicals and reagents

Unless indicated otherwise, chemicals of the highest analytical grade were purchased from Sigma-Aldrich (Dorset, England, UK). All experimental procedures involving the use of live animals were approved under the Animals (Scientific Procedures) Act, 1986 (UK).

Primary cultures of alveolar epithelia

Fetal alveolar type II (fATII) epithelial cells were isolated from lungs of fetuses, essentially as reported elsewhere. Briefly, fetal rats were removed from pregnant Sprague-Dawley rats by caesarian section at day 19 of gestation (term = 22 days), the lungs excised, teased free from heart and upper airway tissue, and were finely minced then washed free of erythrocytes using sterile, chilled Mg2+- and Ca2+-free Hank’s balanced salt solution (HBSS; 0.5 ml/amp). The solution was then centrifuged at 1000 g for 5 min each and then seeded onto 24 mm diameter Transwell-clear permeable supports (Costar; 0.4 μm pore size) at a density of 5 × 104 cells per filter and were allowed to adhere overnight at 152 Torr (≈ 21% O2/5% CO2). DMEM/FCS was exchanged for 4 ml of serum free PC-1 media (Biowhittaker, MD, USA) pre-equilibrated to pH 7.2 and 37°C 24 h later and cells were maintained at this pH until the experiment. In each case, and under conditions of independent treatments, the adenylate energy charge, an index of cell viability and competence, remained ≥ 0.7 and transepithelial monolayer resistance was monitored constantly at 250–350 Ωcm2 or more.

rmTNF-α treatment, western analysis and electrophoretic mobility shift assay

Time-dependent analysis (0–48 h) of the effect of recombinant murine TNF-α (rmTNF-α; 10 ng/ml) on IκB-α/NF-κB signaling was performed. For the dose-response curve, cells were treated with rmTNF-α (0–30 ng/ml) for 0.5 h and filters were twice washed with pre-equilibrated PC-1 medium. Following treatments, sub-cellular extracts (cytosolic and nuclear) were prepared essentially as previously described. Briefly, filters were washed twice in 5 ml ice-cold, pre-equilibrated phosphate buffered saline (PBS; pH 7.2–7.4) and cells (1–2 × 107) were collected and centrifuged at 420 × g for 5 min at 4°C. Nuclei were released by suspending the centrifuged pellet in 250 μl buffer A (lysis buffer) containing (in mM): 10 Tris-HCl (pH 7.8), 10 KCl, 2.5 NaH2PO4, 1.5 MgCl2, 1 NaVO4, 0.5 dithiothreitol (DTT), 0.4 4-2-aminoethyl-benzene sulfonyl fluoride-HCl (AEBSF), and 2 μg/ml each of leupeptin, pepstatin A and aprotinin. The suspension was left in ice for 10 min followed by a 45 s homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at 4200 × g for 5 min at 4°C and re-suspending in 20 μl buffer B (buffer A adjusted to (in mM): 20 Tris-HCl (pH 7.8), 420 KCl, 20% (v/v) Glycerol). The supernatant thus formed is termed the cytosolic extract. The nuclei were then lysed at 4°C for 30 min with gentle agitation, the debris cleared by centrifugation at 10 000 × g for an additional 30 min at 4°C and the supernatant frozen in liquid nitrogen and stored at −70°C until used. In all cases, protein contents were determined by the Bradford method using BSA as a standard. Cytosolic and nuclear proteins (20–25 mg) were resolved over SDS-PAGE (7.5%) gels at RT, blotted onto nitrocellulose membrane, and non-specific binding sites were subsequently blocked. Mouse monoclonal IgG, anti-IκB-α (H-4), IgG2b anti-(phosphorylated) p65 (B-9), rabbit polyclonal IgG anti-p30 (NLS), anti-p52 (K-27), anti-p65 (RelA; A), anti-p68 (RelB; C-19), and anti-p75 (e-Rel; N) (Santa Cruz Biotechnology, UK) antibodies were used for primary detection. Anti-rabbit IgG-biotinylated antibody (Amersham Life Science, UK) was employed for secondary detection followed by the addition of streptavidin-HRP conjugate and visualized on film by chemiluminescence. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable.
Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of β-actin was subsequently performed.\textsuperscript{2,3,22} Custom deoxy-oligonucleotide probe sequences were purchased from Genosys, UK: NF-κB, 5′-AGTTGAGG GGACTTTCCAGGC-3′ (binding sequence underlined). Gel-purified double-stranded DNA was end-labeled with \([\gamma^32P]-ATP\) (NEN Life Sciences Products, UK). Identical amounts of radioactive probe (1–2 × 10\(^4\) counts min \(^{-1}\)) were added to binding reactions containing 1–5 μg \(\gamma\)TII nuclear extracts in a final volume of 40 μl in DNA binding buffer (20 mM HEPES (pH 7.9); 1 mM MgCl\(_2\); 4% Ficoll). Reaction mixtures were incubated for 30 min at 25°C before separating on non-denaturing 4% polyacrylamide gels at RT and subjected to electrophoresis with 1:10 5x Tris-Borate-EDTA buffer. A non-specific competitive deoxyoligonucleotidodeoxyctydilic acid [poly(dI-dC)] (Amersham Pharmacia Biotech, UK) was added to reaction mixtures after addition of labeled probe. Gels were transferred to ion-exchange chromatography paper, vacuum dried and then electronically visualized on a Packard Instant phosphorimag. Specific quantification of the corresponding DNA gel shift bands was performed with phosphorimag.\textsuperscript{2,3,22}

Assessment of pro- and anti-inflammatory cytokines by enzyme-linked immunosorbent assay

The bioactivity of extracellularly released cytokines was measured by a two-site, solid-phase, developed sandwich enzyme-linked immunosorbent assay (ELISA).\textsuperscript{4,5} Immunopair affinity purified polyclonal rabbit anti-rat IL-1β, IL-6, IL-10 and TNF-α (2 μg/ml) primary antibodies were used to coat high-binding microtiter plates (MaxiSorp, Nunc, UK). Recombinant rat and biotinylated immunopair affinity purified sheep anti-rat cytokine (R&D Systems) were used as standard and recognition antibodies, respectively. The color was developed incorporating streptavidin-poly-horseradish peroxidase coupled reaction with the chromagen 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB), and the optical density (O.D.) was measured at 450 nm. Inter- and intra-assay coefficients of variations (CV) were <10%, and the minimum detectable sensitivity for each cytokine is ≤2 pg/ml. Results interpolated from the linear regression of the standard curve are expressed as pg/ml.\textsuperscript{4,5}

Statistical analysis and data presentation

Data are the means and the error bars the SEM. Statistical evaluation of the difference in mean separation was performed by one-way analysis of variance (ANOVA), followed by post hoc Tukey’s test, and the a priori level of significance at 95% confidence level was considered at \(P \leq 0.05\).

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