Possible new role for NF-κB in the resolution of inflammation

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Inflammation involves the sequential activation of signaling pathways leading to the production of both pro- and anti-inflammatory mediators. Although much attention has focused on pro-inflammatory pathways that initiate inflammation, relatively little is known about the mechanisms that switch off inflammation and resolve the inflammatory response. The transcription factor NF-κB is thought to have a central role in the induction of pro-inflammatory gene expression and has attracted interest as a new target for the treatment of inflammatory disease. We show here that NF-κB activation in leukocytes recruited during the onset of inflammation is associated with pro-inflammatory gene expression, whereas such activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis. Inhibition of NF-κB during the resolution of inflammation protracts the inflammatory response and prevents apoptosis. This suggests that NF-κB has an anti-inflammatory role *in vivo* involving the regulation of inflammatory resolution.

The inflammatory response involves the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and release further mediators. This response is self-limiting and resolves through the release of endogenous anti-inflammatory mediators and the clearance of inflammatory cells. The persistent accumulation and activation of leukocytes is a hallmark of chronic inflammation. Current approaches to the treatment of inflammation rely on the inhibition of pro-inflammatory mediator production and of mechanisms that initiate the inflammatory response. However, the mechanisms by which the inflammatory response resolves might provide new targets in the treatment of chronic inflammation. Studies in different experimental models of resolving inflammation have identified several putative mechanisms and mediators of inflammatory resolution. We have shown that cyclopentenone prostaglandins (cyPGs) may be endogenous anti-inflammatory mediators and promote the resolution of inflammation *in vivo*[1]. Others have shown a temporal shift to the production of anti-inflammatory lipoxins during the resolution of inflammation[2]. In recent years, apoptosis has been identified as an important mechanism for the resolution of inflammation *in vivo*[3,4]. It has been postulated that defects in leukocyte apoptosis are important in the pathogenesis of inflammatory disease. In addition, the selective induction of apoptosis in leukocytes may offer a new therapeutic approach to inflammatory disease.

Nuclear factor-κB (NF-κB) is a generic term for a dimeric transcription factor formed by the hetero- or homodimerization of proteins from the rel family, reviewed recently[5]. There are five rel proteins: RelA (p65), RelB and cRel, which contain transactivation domains, and p50 and p52, which are expressed as the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. These precursors require post-translational processing and do not contain transactivation domains. NF-κB is thought to play a pivotal role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS)[5–10]. NF-κB is usually kept inactive in the cytoplasm through association with an endogenous inhibitor protein of the IκB (inhibitor of NF-κB) family. The consensus pathway for NF-κB activation in response to pro-inflammatory stimuli such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) has been extensively characterized[5]. These cytokines act through distinct signaling pathways that converge on the activation of an IκB kinase (IKK); the subsequent phosphorylation of IκB molecules targets them for degradation by the proteosome. IKK consists of three subunits, α, β and γ; IKK-β is responsible for NF-κB activation in response to pro-inflammatory stimuli. Previous studies of mice genetically deficient in components of the NF-κB pathway have shown that Rel proteins are important in the recruitment of leukocytes in the innate immune response. RelA has a role in the non-hematopoietic compartment[11], whereas cRel has a role in promoting hematopoietic cell survival and production of mediators that maintain the inflammatory response[12–14]. Similar studies have shown a role for p50 in negative regulation of macrophage activation[15,16].

Rat carrageenin-induced pleurisy is an established model of acute inflammation, characterized by sequential release of inflammatory mediators and a recruitment of leukocytes that peaks at 24 h and resolves by 48 h (ref. 1). The cellular infiltrate is initially dominated by neutrophil granulocytes, with an increasing percentage of mononuclear phagocytes as inflammation progresses, and with these dominating during resolution. The onset of inflammation is associated with expression of COX2 and iNOS and production of the pro-inflammatory mediators prostaglandin E_2 (PGE_2) and nitric oxide (NO)[17]. The resolution of inflammation is associated with a further increase in COX2 expression, without PGE_2 production or iNOS expression but accompanied by the production of the anti-inflammatory cyPG 15-deoxy-$\Delta^{[12,14]}PGJ_2$ ($15dPGJ_2$) and its precursor PGD_2 (ref. 1). Here we investigate NF-κB activation in leukocytes during the

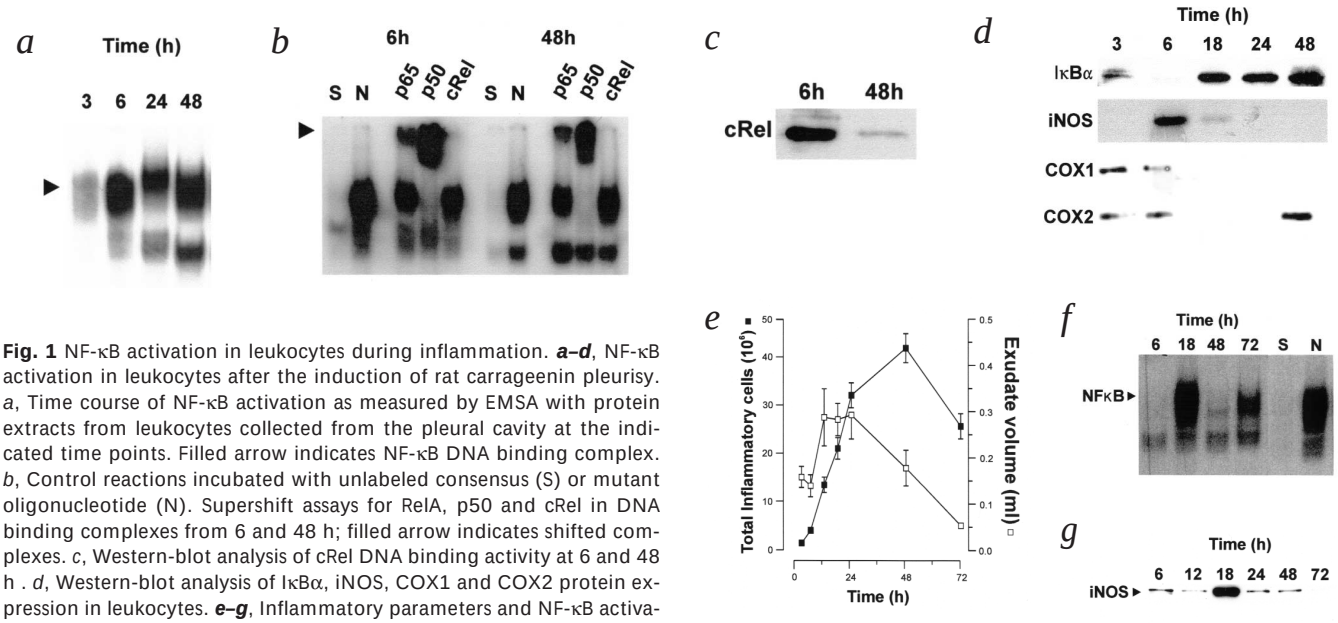


Fig. 1 NF-κB activation in leukocytes during inflammation. ***a–d***, NF-κB activation in leukocytes after the induction of rat carrageenin pleurisy. *a*, Time course of NF-κB activation as measured by EMSA with protein extracts from leukocytes collected from the pleural cavity at the indicated time points. Filled arrow indicates NF-κB DNA binding complex. *b*, Control reactions incubated with unlabeled consensus (S) or mutant oligonucleotide (N). Supershift assays for RelA, p50 and cRel in DNA binding complexes from 6 and 48 h; filled arrow indicates shifted complexes. *c*, Western-blot analysis of cRel DNA binding activity at 6 and 48 h . *d*, Western-blot analysis of IκBα, iNOS, COX1 and COX2 protein expression in leukocytes. ***e–g***, Inflammatory parameters and NF-κB activation in the mouse carrageenin air pouch. *e*, Time course of exudate formation (open boxes) and leukocyte accumulation (closed boxes). Data represented as mean ± s.e.m. (n = 8–10). *f*, Time course of NF-κB activation in leukocytes. Control reactions incubated with unlabeled consensus (S) or mutant oligonucleotide (N). Filled arrow indicates NF-κB DNA binding complex. *g*, Western-blot analysis of iNOS protein expression in leukocytes.

evolution of the inflammatory response. We show NF-κB activation associated with both the onset and the resolution of inflammation in both rat carrageenin pleurisy and mouse carrageenin air pouch. During resolution, however, NF-κB activity is not associated with iNOS expression or the release of pro-inflammatory mediators but is associated with the expression of endogenous anti-inflammatory pathways and leukocyte apoptosis. In addition, inhibition of NF-κB during the resolution of inflammation *in vivo* protracts the inflammatory response and prevents clearance of leukocytes.

NF-κB activation during the resolution of inflammation *in vivo*

We measured the DNA-binding activity of NF-κB in leukocytes from rat carrageenin pleurisy by the electrophoretic mobility shift assay (EMSA) over a 48-hour time course. NF-κB activation in this model was biphasic (Fig. 1*a*). DNA binding activity was initially detected at 6 h during the onset of inflammation and was coincident with iNOS protein expression and the degradation of IκBα protein (Fig. 1*d*). NF-κB DNA binding activity then further increased at 24 and 48 h in the presence of IκBα protein and the absence of iNOS expression. This late-phase activation of NF-κB was associated with the expression of COX2 (Fig. 1*d*). Control experiments using excess unlabeled consensus or mutant κB oligonucleotides indicated that the complexes detected at both 6 and 48 h were specific for the κB binding site (Fig. 1*b*). The addition of antibodies to the p50 subunit of NF-κB showed a strong shift in the mobility of the complexes at both 6 and 48 h (Fig. 1*b*). The addition of RelA antiserum produced a weaker shift in the mobility of complexes at both time points, indicating some DNA-binding of RelA-p50 heterodimers at both 6 and 48 h (Fig. 1*b*). Addition of cRel antiserum seemed to reduce the intensity of the DNA-binding activity at 6 h, but with no visible shift in the mobility of the complex; however, cRel antiserum had no effect on

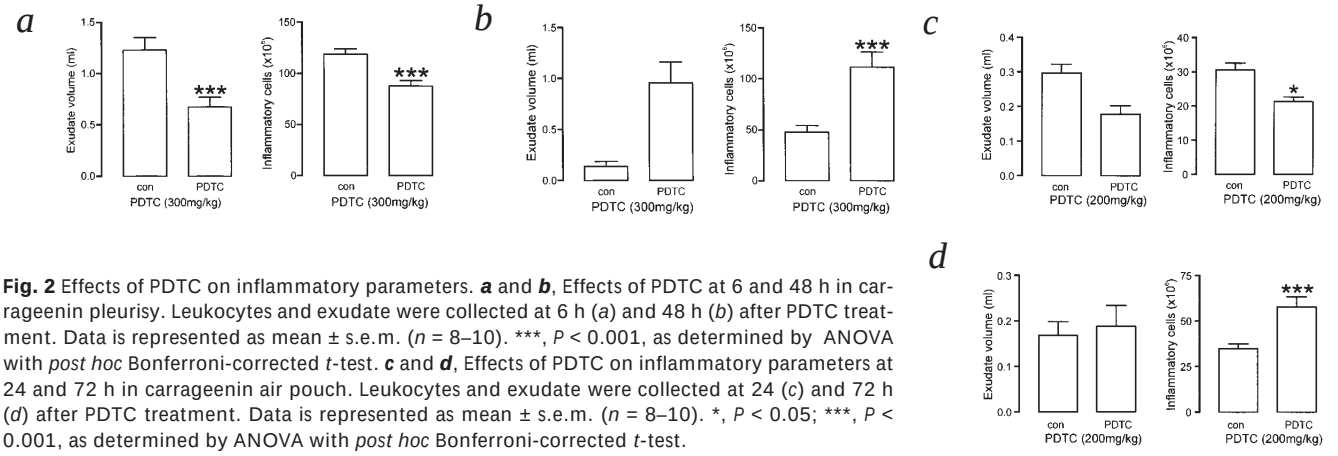


Fig. 2 Effects of PDTC on inflammatory parameters. ***a*** and ***b***, Effects of PDTC at 6 and 48 h in carrageenin pleurisy. Leukocytes and exudate were collected at 6 h (*a*) and 48 h (*b*) after PDTC treatment. Data is represented as mean ± s.e.m. (n = 8–10). ***, $P < 0.001$, as determined by ANOVA with *post hoc* Bonferroni-corrected *t*-test. ***c*** and ***d***, Effects of PDTC on inflammatory parameters at 24 and 72 h in carrageenin air pouch. Leukocytes and exudate were collected at 24 (*c*) and 72 h (*d*) after PDTC treatment. Data is represented as mean ± s.e.m. (n = 8–10). *, $P < 0.05$; ***, $P < 0.001$, as determined by ANOVA with *post hoc* Bonferroni-corrected *t*-test.

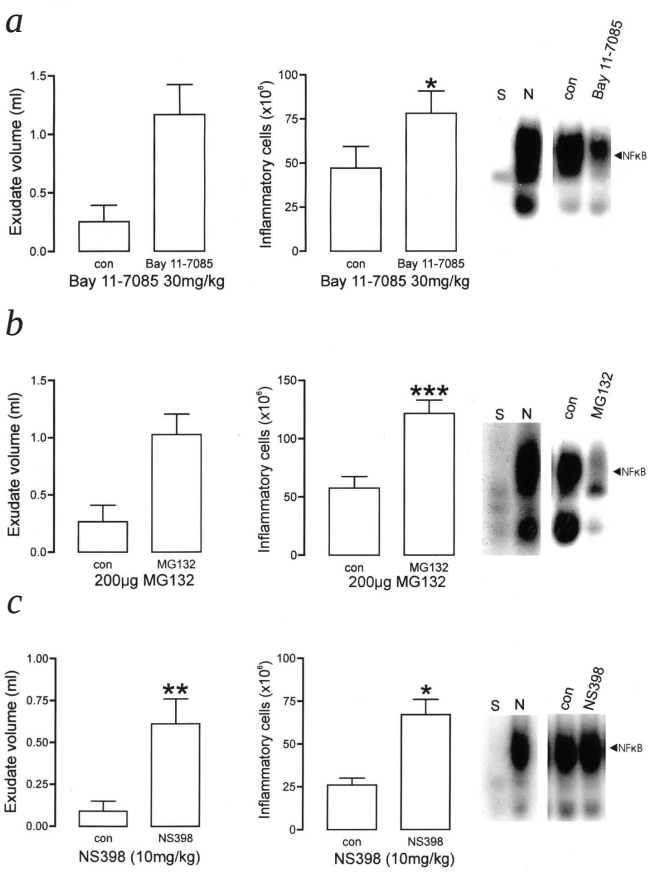


Fig. 3 Inhibition of NF-kB or COX2 activity protracts the inflammatory response *in vivo*. Shown are effects on inflammatory parameters and NF-kB activation determined as described in Figs. 1 and 2 of Bay 11-7085 (***a***) MG132 (***b***) and NS398 (***c***) given during the resolution of inflammation in rat carrageenin pleurisy. Data is represented as mean ± s.e.m. (n = 8–10). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as determined by ANOVA with *post hoc* Bonferroni-corrected t-test.

DNA-binding activity at 48 h (Fig. 1*b*). Antibodies to RelB and p52 did not produce any change in the intensity of DNA binding or the mobility of the complexes at 6 or 48 h (not shown). Western-blot analysis of proteins binding an agarose-conjugated κB response element showed cRel binding activity in cell extracts obtained at 6 h but not at 48 h (Fig. 1*c*). Western-blot analysis did not detect RelB or p52 DNA-binding activity at either 6 or 48 h (not shown). This suggested a shift from a predominance of cRel-p50 complexes at 6 h to a predominance of p50–p50 complexes at 48 h.

Effects of PDTC in rat carrageenin pleurisy

The NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was anti-inflammatory during the onset of the inflammatory response when given prophylactically with carrageenin at 300 mg/kg intraperitoneally (i.p.). The treatment reduced exudate formation by 45% ($P < 0.001$) and the number of leukocytes by 26% ($P < 0.001$) as measured 6 h after carrageenin challenge (Fig. 2*a*). When given therapeutically 24 h after carrageenin challenge, however, PDTC protracted the inflammatory response, inhibiting the resolution of both exudate and leukocytes. Treatment with PDTC led to a 7-fold increase in exudate volume and a 2.3-fold increase in leukocytes ($P < 0.001$) at 48 h (Fig. 2*b*).

NF-κB activation and PDTC in mouse carrageenin air pouch

The observations in rat carrageenin pleurisy were confirmed in a different species and model, the mouse carrageenin air pouch. The carrageenin-induced air pouch is another well-established model of inflammation, characterized by formation of fluid exudate and leukocyte recruitment that begin to resolve at 72 h (Fig. 1*e*). As for the rat model, the cellular infiltrate is initially dominated by neutrophils, with an increasing percentage of mononuclear cells as inflammation progresses and with these dominating during resolution. NF-κB activation in leukocytes from the carrageenin air pouch followed a biphasic pattern similar to that in rat carrageenin pleurisy. NF-κB activation at 18 h (Fig. 1*f*) was associated with the expression of iNOS protein in leukocytes (Fig. 1*g*), whereas NF-κB activity occurred during the resolution of inflammation in the absence of iNOS protein expression. PDTC (200 mg/kg i.p.) was also anti-inflammatory during the onset of inflammation when given prophylactically, reducing exudate formation by 40% and cell accumulation by 30% ($P < 0.05$) 24 h after carrageenin injection (Fig. 2*c*). PDTC given therapeutically 48 h after carrageenin inhibited the resolution of inflammation

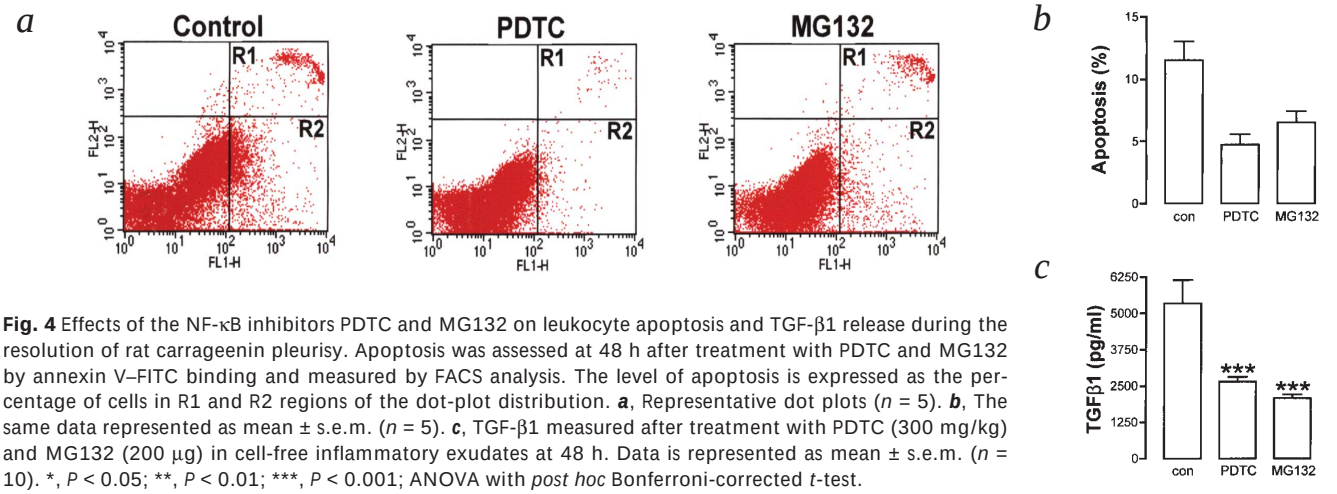


Fig. 4 Effects of the NF-κB inhibitors PDTC and MG132 on leukocyte apoptosis and TGF-β1 release during the resolution of rat carrageenin pleurisy. Apoptosis was assessed at 48 h after treatment with PDTC and MG132 by annexin V–FITC binding and measured by FACS analysis. The level of apoptosis is expressed as the percentage of cells in R1 and R2 regions of the dot-plot distribution. ***a***, Representative dot plots (n = 5). ***b***, The same data represented as mean ± s.e.m. (n = 5). ***c***, TGF-β1 measured after treatment with PDTC (300 mg/kg) and MG132 (200 µg) in cell-free inflammatory exudates at 48 h. Data is represented as mean ± s.e.m. (n = 10). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ANOVA with *post hoc* Bonferroni-corrected t-test.

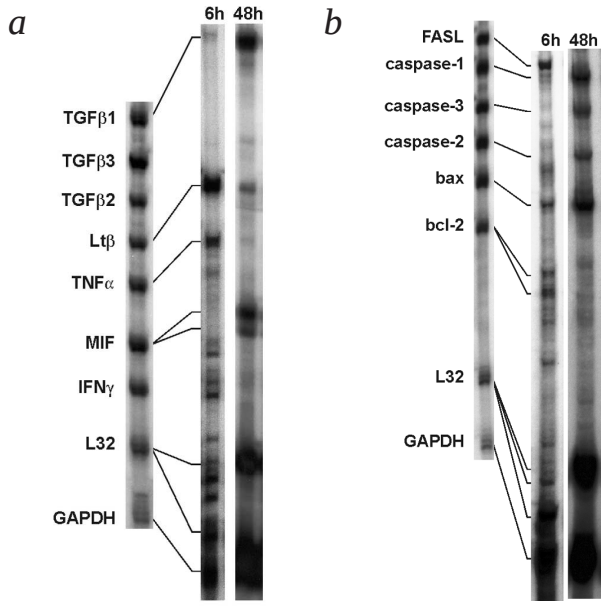


Fig. 5 Analysis of cytokine and apoptosis related gene expression in carrageenin pleurisy. RNase protection assays for cytokine- (***a***) and apoptosis-related (***b***) gene expression with RNA isolated from cells at 6 and 48 h in carrageenin pleurisy. Representative samples of at least 3 independent experiments are shown.

at 72 h, resulting in a 1.7-fold increase in the number of leukocytes ($P < 0.001$) compared to controls, although the volume of exudate was only slightly increased (Fig. 2*d*).

Inhibition of NF-κB DNA binding protracts inflammation

To confirm the results obtained with the broad-spectrum NF-κB inhibitor PDTC, we used two other inhibitors of NF-κB with distinct mechanisms and routes of administration: Bay 11-7085 and the proteosome inhibitor MG132. Both inhibited the DNA-binding activity of NF-κB in leukocytes from the pleural cavity at 48 h when given therapeutically 24 h after carrageenin. Reduced NF-κB DNA-binding activity was associated with the inhibition of resolution. Bay 11-7085 (30 mg/kg i.p.) increased fluid exudate 4.6-fold and the number of leukocytes 2.1-fold ($P < 0.05$) at 48 h (Fig. 3*a*). MG132 (200 μg locally) given at 24 h increased exudate volume 3.8-fold and the number of leukocytes 2.1-fold ($P < 0.001$) at 48 h (Fig. 3*b*). When given in the absence of carrageenin, these inhibitors did not provoke any measurable inflammatory response. The selective COX2 inhibitor NS398 (10 mg/kg) inhibited the resolution of inflammation at 48 h, increasing exudate volume 6.8-fold ($P < 0.01$) and cell number 2.6-fold ($P < 0.05$), but this treatment did not decrease NF-κB DNA-binding activity in leukocytes (Fig. 3*c*).

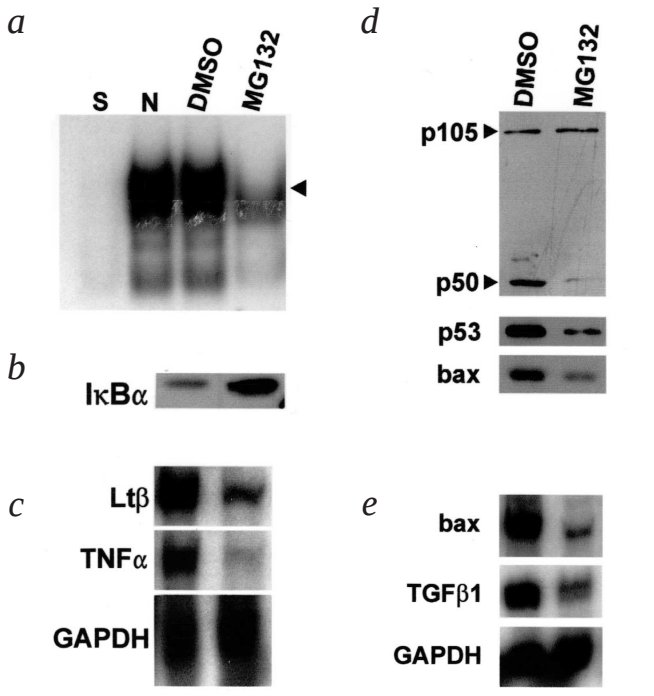
Fig. 6 Effects of MG132 treatment during inflammation. ***a–c***, Effects of 200 μg MG132 on NF-κB activation and pro-inflammatory gene expression at 6 h after the induction of carrageenin pleurisy. Shown are effects on NF-κB DNA-binding activity (*a*), IκBα protein expression (*b*) and TNF-α and Ltβ gene expression (*c*; assessed by RNase protection assay with RNA from cells at 6 h). ***d*** and ***e***, Effects of 200 μg MG132 on p105 processing and expression of p53, Bax and TGF-β1 at 48 h after carrageenin injection. Shown are effects on processing of p105 to p50 (*d*) and the expression of Bax and p53 protein (assessed by western-blot analysis) and Bax and TGF-β1 gene expression (*e*; assessed by RNase protection assay with RNA from cells at 48 h).

NF-κB inhibition reduces leukocyte apoptosis and TGF-β1 release

We assessed apoptosis in inflammatory exudates by annexin V–FITC binding as analyzed by flow cytometry. Cell viability in all treatment groups was more than 92%, as determined by propidium iodide (PI) exclusion. At 48 h in the pleurisy, 11.53 ± 2% of leukocytes were apoptotic, as assessed by annexin V binding to phosphatidylserine (Fig. 4*a*). PDTC and MG132 given at 24 h reduced the number of apoptotic leukocytes at 48 h to 4.74 ± 0.86% and 6.5 ± 0.92%, respectively (Fig. 4*b*). We obtained very similar results with a FACS-based terminal deoxynucleotidyltransferase–mediated dUTP nick end-labeling (TUNEL) assay (not shown). We also measured the anti-inflammatory cytokine transforming growth factor-β1 (TGF-β1) in cell-free pleural exudates by ELISA. Both PDTC and MG132 reduced TGF-β1 release at 48 h, by 50% ($P < 0.001$) and 61% ($P < 0.001$), respectively (Fig. 4*c*).

Inhibition of NF-κB reduces Bax, p53 and TGF-β1 expression *in vivo*

We used RNase protection assays and western-blot analysis to analyze gene expression in leukocytes. The profiles of cytokine- (Fig. 5*a*) and apoptosis-related (Fig. 5*b*) gene expression in leukocytes from the pleural cavity indicate that the related pro-inflammatory cytokines lymphotoxin β (Ltβ) and TNF-α and the anti-apoptotic protein Bcl2 were preferentially expressed at 6 h after carrageenin injection, whereas the anti-inflammatory cytokine TGF-β1 and the pro-apoptotic Bcl2 homolog Bax were expressed at 48 h. Prophylactic treatment with MG132 concurrently with carrageenin inhibited IκBα degradation and NF-κB activation in leukocytes at 6 h (Fig. 6*a* and *b*); this was associated with reduced TNF-α and Ltβ gene expression (Fig. 6*c*). However, therapeutic treatment with MG132 as described above inhibited NF-κB1 (p105) processing to p50 and reduced TGF-β1, Bax and p53 expression in leukocytes at 48 h (Fig. 6*d* and *e*).



Discussion

In the models described here, onset of inflammation is characterized by the release of pro-inflammatory cytokines TNF-α and IL-1β, which signal pro-inflammatory gene expression through IKK-β (ref. 5). Resolution of inflammation is associated with production of COX2 derived anti-inflammatory prostaglandins PGD_2 and $15dPGJ_2$ (ref. 1) and the anti-inflammatory cytokine TGF-β1. Here we show NF-κB activation coincident with iNOS expression during the onset of inflammation in both rat carrageenin pleurisy and mouse carrageenin air pouch, in keeping with previous results[10]. However, we did not detect iNOS expression during the resolution of inflammation despite the presence of substantial NF-κB activation. Although DNA-binding complexes at the onset of inflammation contained both cRel and p50, cRel-binding activity was not detected during the resolution of inflammation. The cRel-p50 heterodimer binds and regulates the mouse iNOS promoter[18] and macrophages from cRel knockout mice show impaired iNOS gene expression[13]. This may provide a mechanistic explanation for the lack of iNOS expression in response to NF-κB activation during the resolution of inflammation *in vivo*.

Signal-induced processing of p105 to p50 is dependent on inducible phosphorylation and proteosome-mediated limited proteolysis, which is dependent in turn on the IKK complex[19]. However, although the IKK complex is essential for inducible p105 processing, IKK-β is redundant in this pathway[19]. Activation of NF-κB at 6 h in carrageenin pleurisy was associated with IκBα degradation as expected given that IKK-β signals IκBα degradation, but NF-κB activation at 48 h was detected in the presence of IκBα. Taking into consideration the high affinity of IκBα for RelA- and cRel-containing complexes, this further suggests that p50–p50 homodimers represent the predominant NF-κB activity at 48 h. Furthermore, cyPGs produced during the resolution of inflammation[1] specifically inhibit IKK-β *in vitro*[20–22]. This may indicate an IKK-β-independent pathway of NF-κB activation *in vivo*. Notably, a recent report has described an IKK-α-dependent pathway for NF-κB activation independent of IKK-β that is associated with a distinct profile of gene expression[23].

The persistence of p50–p50 homodimers during the resolution of inflammation is perhaps not surprising, considering the reported role of p50–p50 in the repression of pro-inflammatory gene transcription[15,16]. Transgenic mice that express p50 but not the inhibitory C-terminus of the p105 molecule show increased p50–p50 DNA-binding activity[16]. These mice show reduced expression of cytokine genes in resident macrophages and impaired leukocyte recruitment, suggesting p50-mediated suppression of inflammation *in vivo*. These studies also showed positive transcriptional effects in certain cell types, however, which may indicate a role for cell-specific cofactors in dictating the outcome of NF-κB activation *in vivo*. Both *in vitro* and *in vivo* studies suggest that p50–p50 may enhance gene transcription, particularly when complexed with the IκB-like protein Bcl3 (refs. 24,25). We were unable to detect Bcl3 in DNA-binding complexes at 48 h; however, this does not exclude the presence or absence of other transcriptional cofactors not of the Rel family that might alter the transcriptional properties of NF-κB. This may be particularly relevant to the expression of COX2 during the resolution of inflammation. RelA-p50 and p50–p50 complexes bind to distinct κB response elements in the COX2 promoter[26]. However, p50–p50 is important in COX2 transcription through association with transcriptional co-activators of the C/EBP family[26,27].

Inhibitors of the NF-κB pathway have anti-inflammatory effects *in vivo*[28–30]. However, during the resolution of inflammation, these agents inhibited NF-κB activation in leukocytes and protracted the inflammatory response. Notably, the selective COX2 inhibitor NS398 did not reduce NF-κB DNA binding activity in leukocytes, which suggests that cyPGs may act downstream of NF-κB in the resolution of inflammation *in vivo*. There are concerns with the use of these inhibitors of NF-κB, which may have effects independent of the NF-κB pathway. However, use of inhibitors allows modulation of NF-κB at specific stages of the inflammatory response, which is central to the findings reported here. It is important to note that such inhibitors may prevent the proper resolution of inflammation *in vivo*. Unfortunately, available molecular genetic tools do not allow modulation of the NF-κB pathway at different stages of the inflammatory response *in vivo*. It is to be hoped that the development of such tools will help to define the role of the NF-κB pathway in inflammation.

Leukocyte apoptosis and subsequent clearance by macrophages is important for the resolution of inflammation[3,4]; in addition, the NF-κB pathway regulates both pro- and anti-apoptotic pathways[31]. We have also shown here that inhibition of NF-κB inhibits apoptosis during the resolution of inflammation *in vivo*. Expression of genes involved in apoptosis, including the pro-apoptotic Bcl-2 homolog Bax and the pro-apoptotic transcription factor p53 (refs. 32,33), was modulated by NF-κB inhibitors in carrageenin pleurisy. Inhibition of the processing of p105 to p50 and of NF-κB DNA binding activity by the proteosome inhibitor MG132 reduced the expression of Bax and p53 in leukocytes at 48 h. It is noteworthy that Bax expression is detected in leukocytes during the resolution of inflammation. Cytokine-mediated repression of Bax might cause the persistence of granulocytes in inflammatory diseases through delayed apoptosis[34]. The pro-apoptotic action of p53 expression in cells of myeloid lineage is also suppressed by pro-inflammatory cytokines[33], and p53 and Bax expression are induced during LPS-induced macrophage apoptosis *in vitro*[35]. In contrast, TNF-α–induced apoptosis, which is dependent on IKK-β activation and IκBα degradation[36], is not associated with expression of Bax and p53[35]. Bax is thought to be a target gene for p53 rather than for NF-κB (refs. 32,33), although the Bax promoter contains both κB and p53 response elements[37]. NF-κB is known to regulate p53 expression, however; in addition, p50–p50 homodimers positively regulate the p53 promoter[38–40]. It is possible that p53 and NF-κB (p50–p50) cooperate to regulate Bax expression and apoptosis.

Previous studies suggest that TGF-β1 release during the resolution of inflammation may occur in response to phagocytosis of apoptotic neutrophils by macrophages[41–43]. We show that NF-κB inhibitors reduce TGF-β1 expression and release by leukocytes during the resolution of inflammation. The NF-κB pathway may regulate leukocyte apoptosis and the subsequent releaseof TGF-β1 during the resolution of inflammation *in vivo*. $15dPGJ_2$ induces apoptosis in activated mouse macrophages *in vitro* while inhibiting iNOS protein expression[44]. Furthermore, $15dPGJ_2$ induces apoptosis and suppresses adjuvant-induced arthritis in rats[45]. CyPGs released during the resolution of inflammation[1] may suppress an anti-apoptotic IKK-β/NF-κB pathway, thereby promoting leukocyte apoptosis and the resolution of inflammation.

We suggest a new role for NF-κB in the resolution of inflammation through the regulation of leukocyte apoptosis. Current

studies on the role of NF-κB and cyPGs in the resolution of inflammation should yield new avenues in inflammation research. The identification of new anti-inflammatory pathways is an exciting prospect. Their elucidation will no doubt provide potential new targets for the treatment of inflammatory disease.

Methods

Carrageenin-induced pleurisy. Male Wistar rats (150 ± 20 g in body weight; Tuck and Sons, Battlesbridge, UK) were treated with 0.15 ml of 1% (w/v) λ-carrageenin (Sigma-Aldrich, Poole, UK) injected into the pleural cavity and inflammatory exudates were collected as described[1]. All animal experimentation was done according to the appropriate regulations for the care and use of animals.

Mouse carrageenin air pouch. Three-milliliter air pouches were raised on the dorsal surface of female outbred Swiss albino mice (28 ± 2 g in body weight; Tuck and Sons) under light halothane anesthesia, and a further 1.5 ml of air was injected 4 d later. At day 7, 0.5 ml of 1% (w/v) λ-carrageenin was injected into the pouch. Inflammatory exudates were collected as described above.

Drug treatments. PDTC in physiological saline and Bay 11-7085 (Alexis Corporation, Nottingham, UK) in 20% (v/v) PEG 400 with 5% (w/v) BSA in physiological saline were administered i.p. concurrently with carrageenin or 24 h after carrageenin in the pleurisy and 48 h after carrageenin in the air pouch. NS398 (Alexis) was administered orally in gum tragacanth (1% (w/v) in tap water) at 24 h and every 6 h thereafter. MG132 (Alexis) in 10 μl DMSO was injected into the pleural cavity concurrently or 24 h after carrageenin. Control groups received the appropriate vehicle.

Electrophoretic mobility-shift assay (EMSA). Leukocytes from pleural or air-pouch exudates were lysed in buffer containing 20 mM HEPES (pH 7.9), 350 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 20% (v/v) glycerol, 0.6% (v/v) Nonidet P-40 and 5 mM DTT. This included a cocktail of protease inhibitors consisting of PMSF (0.5 mM), leupeptin (0.5 μg/ml), protease inhibitor (0.5 μg/ml), trypsin inhibitor (1 μg/ml), aprotinin (0.5 μg/ml) and bestatin (40 μg/ml). NF-κB consensus (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and mutant oligonucleotides (5′-AGTTGAGGCGACTTT CCCAGGC-3′) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Oligonucleotides were end labeled with [γ-^{32}P]ATP (ICN Biochemicals, Oxfordshire, UK) using T4 polynucleotide kinase (Promega, Southampton, UK). Binding reactions were prepared with 20 μg of protein extract in binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, 0.5 μg poly dI•dC (Becton Dickinson, Oxford, UK), 2.5% (v/v) glycerol and 2% (v/v) Ficoll) to final volume of 20 μl. Supershift reactions included 1–5 μg of antiserum to either RelA (P65), p50, p52, c-Rel or RelB. Consensus and mutant competitor oligonucleotides (1.75 pmol) were added to appropriate controls. Reactions were incubated on ice for 10 min, 0.035 pmol of ^{32}P-labeled oligonucleotide was added and the reactions were incubated on ice for a further 20 min. Binding reactions were electrophoresed on 5% non-denaturing polyacrylamide gels and analyzed by autoradiography.

Western-blot analysis. Protein extracts were diluted 1:1 in loading buffer (125 mM Tris-HCl (pH 7.2), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 2 mM EDTA and 1 mg/ml Coomassie blue) and boiled for 5 min. The extracts were then separated (20-μg aliquots) on 7.5% (iNOS, p100-p52, p105-50), 10% (p53, COX1, COX2, cRel, RelB, p65) or 15% (IκBα, Bax) polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibodies and horseradish-peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Blots were developed with chemiluminescent Luminol reagent (Santa Cruz Biotechnology). DNA binding activity of Rel proteins was measured in cell extracts by western blotting. DNA binding proteins were precipitated from 250–500 μg of protein extract with agarose-conjugated κB oligonucleotides (Santa Cruz Biotechnology) in the presence of 1 μg/ml poly dI•dC and western blotting carried out as above.

Assessment of leukocyte apoptosis. Apoptosis was determined using an annexin V–FITC apoptosis detection kit and viability was assessed by propidium iodide exclusion, done according to the manufacturer's instructions (Sigma-Aldrich). Labeled cells were separated and quantified by flow cytometry with a FACScan (Becton Dickinson) and the data was analyzed using CELLQuest software.

TGF-β1 determination. TGF-β1 was assayed in cell-free inflammatory exudates by commercial ELISA (Quantakine TGF-β1 sandwich ELISA, R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

RNase protection assays. Total cellular RNA was isolated with Trizol reagent, and 20 μg of RNA was used for multiprobe RNase protection assays with Riboquant template sets (BD Pharmingen) done according to the manufacturer's instructions.

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