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Gerry, A. B. and Leake, D. S. ORCID: <https://orcid.org/0000-0002-1742-6134> (2014) Effect of low extracellular pH on NF- $\kappa$ B activation in macrophages. *Atherosclerosis*, 233 (2). pp. 537-544. ISSN 0021-9150 doi: 10.1016/j.atherosclerosis.2014.01.014 Available at <https://centaur.reading.ac.uk/36330/>

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Published version at: <http://dx.doi.org/10.1016/j.atherosclerosis.2014.01.014>

To link to this article DOI: <http://dx.doi.org/10.1016/j.atherosclerosis.2014.01.014>

Publisher: Elsevier

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# Effect of low extracellular pH on NF- $\kappa$ B activation in macrophages<sup>☆</sup>



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## ARTICLE INFO

### Article history:

Received 31 May 2013

Received in revised form

12 December 2013

Accepted 8 January 2014

Available online 21 January 2014

### Keywords:

Atherosclerosis

Cytokines

Inflammation

Low pH

Macrophages

NF- $\kappa$ B

p50 Homodimers

## ABSTRACT

**Objective:** Many diseases, including atherosclerosis, involve chronic inflammation. The master transcription factor for inflammation is NF- $\kappa$ B. Inflammatory sites have a low extracellular pH. Our objective was to demonstrate the effect of pH on NF- $\kappa$ B activation and cytokine secretion.

**Methods:** Mouse J774 macrophages or human THP-1 or monocyte-derived macrophages were incubated at pH 7.0–7.4 and inflammatory cytokine secretion and NF- $\kappa$ B activity were measured.

**Results:** A pH of 7.0 greatly decreased pro-inflammatory cytokine secretion (TNF or IL-6) by J774 macrophages, but not THP-1 or human monocyte-derived macrophages. Upon stimulation of mouse macrophages, the levels of I $\kappa$ B $\alpha$ , which inhibits NF- $\kappa$ B, fell but low pH prevented its later increase, which normally restores the baseline activity of NF- $\kappa$ B, even though the levels of mRNA for I $\kappa$ B $\alpha$  were increased. pH 7.0 greatly increased and prolonged NF- $\kappa$ B binding to its consensus promoter sequence, especially the anti-inflammatory p50:p50 homodimers. Human p50 was overexpressed using adenovirus in THP-1 macrophages and monocyte-derived macrophages to see if it would confer pH sensitivity to NF- $\kappa$ B activity in human cells. Overexpression of p50 increased p50:p50 DNA-binding and in THP-1 macrophages inhibited considerably TNF and IL-6 secretion, but there was still no effect of pH on p50:p50 DNA binding or cytokine secretion.

**Conclusion:** A modest decrease in pH can sometimes have marked effects on NF- $\kappa$ B activation and cytokine secretion and might be one reason to explain why mice normally develop less atherosclerosis than do humans.

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## 1. Introduction

Atherosclerosis is a chronic inflammatory disease [1]. Although large atherosclerotic plaques may cause a degree of stenosis and tissue ischaemia, the majority of clinical endpoints of atherosclerotic disease, such as myocardial infarction or stroke, arise from the exposure of the thrombogenic contents of atherosclerotic lesions to the circulation, thus giving rise to thrombi or emboli [2]. Thus plaque type or composition, as well as plaque size, are important. It is generally understood that those lesions that are prone to rupture with a weakness of the fibrous cap are those that have more areas of low extracellular pH, high lipid content, and a more

inflammatory phenotype with the presence of large numbers of macrophage [2].

Like other focal inflammatory situations, such as rheumatoid arthritis [3] and tumours [4], atherosclerotic lesions have regions of low extracellular pH [5–7]. The pH across atherosclerotic lesions is heterogeneous and may vary by up to a whole pH unit, from pH 7.8 to as low as pH 6.8, presumably reflecting the presence of activated macrophages in the lipid-rich areas and alkali calcium salts in the calcified areas [6]. A few studies have addressed the role of low extracellular pH on atherogenesis, for instance, a low pH can promote the oxidation of low density lipoprotein (LDL) by macrophages [8], some antioxidants work less well at a lower pH [9], oxidised LDL causes less apoptosis of macrophages at low pH [10] and a low pH decreases cholesterol release from macrophages [11]. Additionally, chronic acidosis in end stage chronic renal disease is associated with accelerated atherosclerosis and cardiovascular morbidity and mortality [12].

Nuclear factor (NF)- $\kappa$ B is a transcription factor pivotal for the development and control of inflammation. Dysregulation of NF- $\kappa$ B signalling has been shown to play a major role in many chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and atherosclerosis [13–16].

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The mammalian NF- $\kappa$ B (Rel) family consists of hetero- and homodimers of p65 (RelA), p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), RelB and c-Rel. The p50 and p52 subunits are produced by processing of their precursor molecules p105 and p100, respectively, and lack trans-activation domains [17]. Classical NF- $\kappa$ B, composed of heterodimers of p65 and p50, is a potent activator of a plethora of generally pro-inflammatory cytokines, such as interleukin (IL)-6 and tumour necrosis factor (TNF). In an inactive cell, the classical p65:p50 dimer is held in the cytosol in an inactive state bound to its inhibitory protein I $\kappa$ B $\alpha$ . In response to a variety of extracellular molecules, such as lipopolysaccharide (LPS), IL-1 $\beta$  or TNF, I $\kappa$ B $\alpha$  is phosphorylated by I $\kappa$ B kinase (IKK) complex and targeted for degradation by the 26S proteasome. The p65:p50 dimer is then free to translocate to the nucleus to regulate gene expression. One of the genes it activates is that for I $\kappa$ B $\alpha$ , which inhibits NF- $\kappa$ B in a negative feedback loop [17].

Less well known, however, is NF- $\kappa$ B signalling by the alternative p50 homodimer. In contrast to p65:p50 heterodimers, p50 homodimers are thought usually to be transcriptional repressors due to p50, unlike most other Rel family members, lacking a trans-activation domain [18,19]. p50 thus requires the formation of heterodimers [20] or binding with other transcriptional activators, such as Bcl3 [21] to be transcriptionally active.

NF- $\kappa$ B signalling has complex roles in atherosclerosis [22–24]. Inhibition of NF- $\kappa$ B activation by a macrophage-restricted depletion of IKK2 surprisingly led to an increase in atherosclerotic lesion size and severity in LDL receptor knockout mice [25], but in another study with these mice its myeloid-specific deficiency decreased atherosclerosis [26]. An NF- $\kappa$ B decoy oligonucleotide inhibited neointima formation in rats [27]. The knockdown of IKK $\epsilon$  decreased atherosclerosis [28], as did a cell-permeable peptide that inhibited the nuclear import of NF- $\kappa$ B [29], the NF- $\kappa$ B inhibitor dehydroxymethyllepoxyquinomicin [30] and the endothelial-specific inhibition of NF- $\kappa$ B [31] in mice. Haemopoietic deficiency of the gene encoding for p50 led to smaller, but more inflammatory, lesions in mice [32].

IL-10, produced by many cell types including monocytes/macrophages, is a pleiotropic cytokine that suppresses the production of macrophage inflammatory proteins such as IL-6 and TNF [33,34]. IL-10 has been demonstrated to block NF- $\kappa$ B pro-inflammatory signalling through the suppression of IKK [35] and by inducing the expression of BCL-3, which can inhibit NF- $\kappa$ B activity [36]. IL-10 causes the selective induction of nuclear translocation and DNA-binding of p50 homodimers [37].

As many chronic inflammatory diseases contain areas of low extracellular pH, we have investigated the effect of pH on NF- $\kappa$ B activity in macrophages. We used a pH of 7.0 because the macrophage-rich regions of human carotid atherosclerotic plaques contain considerable areas with this extracellular pH [6]. We have found that reducing the extracellular pH can inhibit greatly cytokine secretion in murine, but not human, macrophages. We suggest that this inhibition of cytokine secretion in murine cells is due to increased p50 homodimer anti-inflammatory signalling in response to a low pH.

## 2. Methods

### 2.1. Materials

Unless stated otherwise all materials were obtained from Sigma Aldrich (Poole, Dorset, U.K.) in their highest purity. RPMI, Dulbecco's modified Eagle medium (DMEM), phosphate buffered saline (PBS), foetal calf serum (FCS), Glutamax, penicillin and streptomycin were obtained from Invitrogen (Paisley, U.K.). THP-1 and J774 cells were obtained from the European Collection of Animal Cell

Cultures (Salisbury, Wiltshire, U.K.). [ $\gamma$ - $^{32}$ P]-ATP (370MBq/ml, 10 mCi/ml), polyvinylidene difluoride membrane (PVDF) and poly(dI-dC)–poly(dI-dC) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, U.K.). NF- $\kappa$ B Gel Shift Assay System was obtained from Promega (Southampton, Hampshire, U.K.). Goat polyclonal antibodies for p50 (cross reactive with p105), rabbit polyclonal antibodies to p50, p65 or I $\kappa$ B $\alpha$  and actin, donkey anti-goat IgG–horseradish peroxidase (HRP) and supershift antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Donkey anti-goat IgG–fluorescein isothiocyanate (FITC) was obtained from AbD Serotec (Oxford, U.K.). Lymphoprep was obtained from Axis-Shield (Oslo, Norway).

### 2.2. Cell culture

Human peripheral blood monocytes were isolated from the blood of healthy volunteers using Lymphoprep, as described previously [38]. This project has been reviewed by the University of Reading Research Ethics Committee and has been given a favourable opinion for conduct. Monocytes were cultured in RPMI supplemented with 10% (v/v) human serum containing 2 mM Glutamax, penicillin (20 IU/ml) and streptomycin (20  $\mu$ g/ml) for 7–10 days to differentiate into human monocyte-derived macrophages (HMDM). The J774 murine macrophage-like cell line was cultured in DMEM containing glucose (1 g/l), sodium pyruvate and pyridoxine, supplemented with 10% FBS (v/v), 2 mM Glutamax, penicillin (20 IU/ml), streptomycin (20  $\mu$ g/ml) and amphotericin B (0.95  $\mu$ g/ml) and maintained at 37 °C in a humidified 95% air/5% CO<sub>2</sub> atmosphere. THP-1 human monocytes were cultured in RPMI containing 2 mM glutamine, 10% FBS (v/v), penicillin (20 IU/ml) and streptomycin (20  $\mu$ g/ml). THP-1 cells were differentiated prior to treatment by phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) for 48 h. Culture medium pH was modified by the addition of an appropriate volume of 5 M HCl. These flasks of medium were allowed to equilibrate at 37 °C in a humidified 95% air/5% CO<sub>2</sub> atmosphere for at least four days before the pH was determined. Culture medium pH was determined rapidly before, after and during incubation with cells using a Mettler-Toledo MP220 pH meter with an Inlab 423 microelectrode. The pH meter was calibrated using prewarmed standards with the required meter temperature manual adjustments.

### 2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using nuclei lysis solution and the electrophoretic mobility shift assay was performed using the NF- $\kappa$ B Gel Shift Assay System according to the manufacturer's instructions. Supershift reactions were carried out by pre-incubation of nuclear extracts with an excess of p50 antibody (goat polyclonal IgG raised against a peptide mapping inside the nuclear localisation sequence) and p65 antibody (goat polyclonal IgG mapping within the amino terminal domain). Semi-quantitative analysis was performed with Quantity One software (Bio-Rad).

### 2.4. Adenovirus-infection studies

Recombinant adenoviruses were produced from constructs expressing p50 (AdV-p50) or EGFP (AdV-EGFP) generously provided by Dr Nicole Horwood (Imperial College London, U.K.) and Dr Katrina A. Bicknell (Pharmacy, University of Reading, U.K.), respectively. PMA-differentiated THP-1 macrophages or serum-differentiated primary human macrophages were incubated for 72 h with either no virus, AdV-p50 or AdV-EGFP at a multiplicity of infection (MOI) of 100 plaque-forming units of each virus per cell. This MOI was determined as sufficient to achieve as high as possible

overexpression with minimal toxicity. Infection was visualised using fluorescent microscopy. Culture medium was then replaced and the cells treated as required.

### 2.5. Confocal microscopy

PMA-differentiated THP-1 macrophages or serum-differentiated primary human macrophages were grown on glass coverslips before incubation with or without adenovirus. The culture medium was replaced and cells treated as stated in the figure legends. The cells were then washed three times with PBS, fixed and permeabilised with 4% (w/v) paraformaldehyde in PBS at room temperature for 15 min and 100% methanol at  $-20^{\circ}\text{C}$  for 10 min. They were washed twice with PBS containing 1% (w/v) BSA and 0.01% sodium azide. The cells were then incubated with goat polyclonal anti-p50 IgG (5  $\mu\text{g/ml}$ ) for 1 h at room temperature, washed four times with PBS containing 1% (w/v) BSA and 0.01% sodium azide and incubated with FITC- or Cy3-conjugated donkey anti-goat IgG (5  $\mu\text{g/ml}$ ) and propidium iodide for 1 h at room temperature. Coverslips were then mounted on the slides using VECTASHIELD™ mounting medium (Vector Laboratories, U.K.) and sealed with nail varnish before storage at  $4^{\circ}\text{C}$  in the dark. Microscope slides were analysed using a Leica TCS NT confocal microscope (Leica Microsystems) using a  $63\times$  immersion objective lens. Semi-quantification was performed using Leica confocal software.

### 2.6. Western blotting

Whole cell lysates were prepared as described previously [39]. Protein content was estimated as described by Bradford [40]. Protein extracts (20  $\mu\text{g}$  of protein) were separated by SDS-PAGE. Proteins were electrotransferred onto a PVDF membrane using a semi-dry blotter (BioRad, Hertfordshire, U.K.). Detection of p50/p105 (1:400) and actin (1:1000) was achieved using goat polyclonal IgGs followed by incubation with a donkey anti-goat IgG–HRP (1:4000). Detection was completed by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

### 2.7. Cytokine ELISA

Culture medium was collected following incubation of cells at the required pH in the absence or presence of LPS (100  $\mu\text{g/ml}$ ) for the stated times. Cell debris was removed by centrifugation. Samples were assayed for TNF or IL-6 by Quantikine ELISA as stated in the manufacturer's instructions (R&D Systems, Minneapolis, U.S.A.).

### 2.8. Statistical analysis

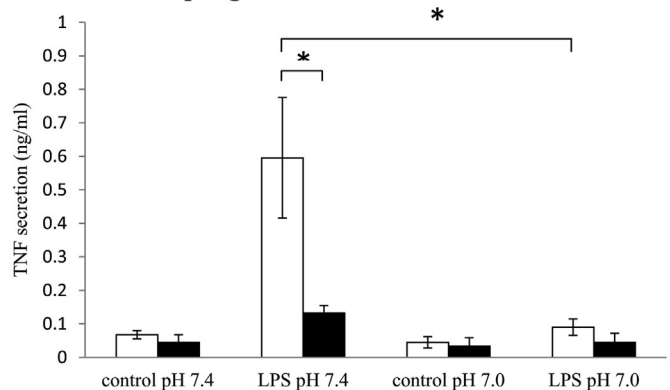
Where indicated graphs represent the mean  $\pm$  S.E.M. of the mean of  $n$  independent experiments. Statistical analysis was performed using ANOVA or the Student's paired  $t$ -test to determine differences between observations. Differences between observations were deemed to be significant when  $P < 0.05$ .

## 3. Experimental results

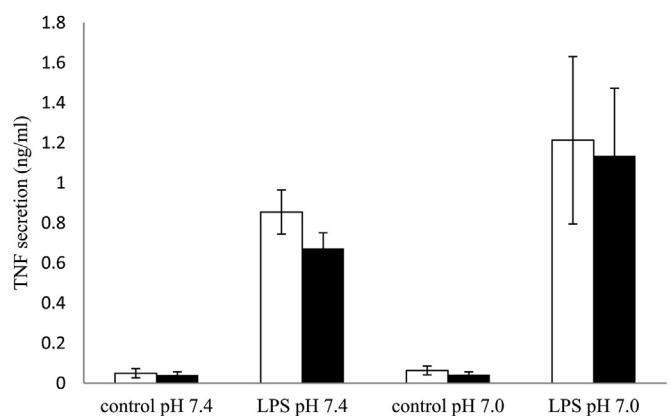
### 3.1. Effects of pH on cytokine secretion by macrophages

As atherosclerotic lesions are known to have a low extracellular pH [6], we investigated the effects of a moderate reduction of pH on the secretion of the inflammatory cytokine TNF by macrophages. LPS greatly increased the secretion of TNF by murine J774 macrophages at pH 7.4 (Fig. 1A). TNF secretion was similar in the absence of LPS at pH 7.0 and 7.4, but TNF secretion in the presence of LPS was reduced by 85% at pH 7.0 compared to pH 7.4. We have shown

### A: J774 macrophages



### B: THP-1 macrophages



**Fig. 1.** Effect of pH on TNF secretion by macrophages. J774 (A) or THP-1 macrophages (B) were incubated at pH 7.4 or 7.0 for 24 h before treatment with or without IL-10 (20 ng/ml) for 2 h followed by LPS (100  $\mu\text{g/ml}$ ) for 2 h. The concentration of TNF was then measured in the medium by ELISA. The white and black bars show the data in the absence or presence of IL-10, respectively. \* $P < 0.05$ .

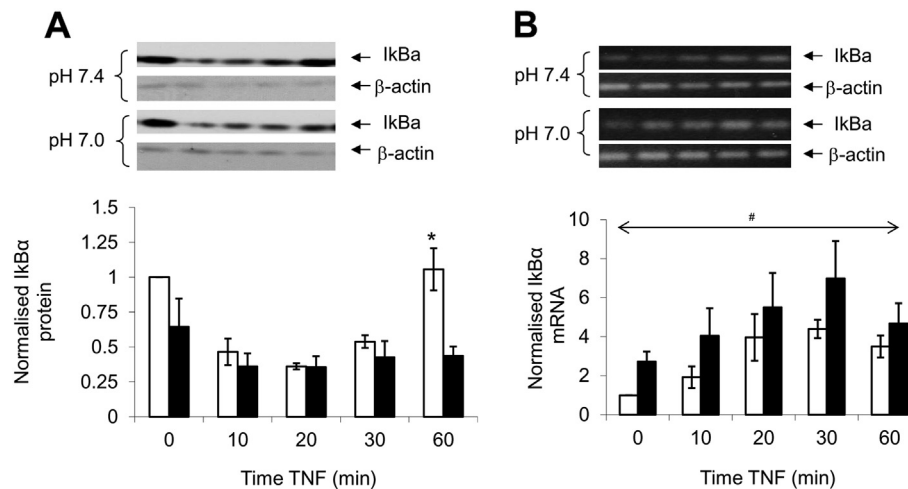
previously that incubation of J774 macrophages for 24–48 h at pH 7.0 is not toxic to the cells [10]. LPS increased greatly the secretion of TNF by human THP-1 macrophages, but the increase was comparable at pH 7.4 and 7.0 (Fig. 1B). Similarly to the effect of low pH, the anti-inflammatory cytokine IL-10 inhibited LPS-induced cytokine secretion by J774 cells, but not by THP-1 cells (Fig. 1).

### 3.2. Effects of pH on I $\kappa$ B $\alpha$ levels in macrophages

To explore the mechanisms of the effects of low pH on cytokine secretion by macrophages, we investigated the effect of pH on the levels of I $\kappa$ B $\alpha$  protein and mRNA. NF- $\kappa$ B is the master transcription factor for inflammatory cytokine production and is normally inhibited by the protein I $\kappa$ B $\alpha$ . When cells are stimulated, I $\kappa$ B $\alpha$  is phosphorylated, polyubiquitinated and degraded by proteasomes, enabling NF- $\kappa$ B to enter the nucleus and increase the transcription of inflammatory genes. We therefore measured the levels of I $\kappa$ B $\alpha$  protein by Western blots in macrophages stimulated by TNF at normal and low pH. At pH 7.4, the levels of I $\kappa$ B $\alpha$  decreased within 10 min, reached the lowest level at 20 min and then started to increase, returning to normal at 60 min (Fig. 2A). At pH 7.0, the levels of I $\kappa$ B $\alpha$  decreased within 10–20 min, but did not increase thereafter.

### 3.3. Effects of pH on I $\kappa$ B $\alpha$ mRNA levels in macrophages

To explore the mechanism for the failure of I $\kappa$ B $\alpha$  to increase after its fall at low pH, we measured the level of mRNA encoding for this



**Fig. 2.** Effect of pH on the levels of IκBα protein and mRNA levels. J774 macrophages were incubated for 24 h at pH 7.4 (white bars) or pH 7.0 (black bars) and then at these pH values for up to 60 min with TNF (25 ng/ml). (A) Shows the normalised levels of IκBα protein relative to actin measured by Western blots. The mean  $\pm$  S.E. of three independent experiments is shown. \* $P < 0.05$  for the difference between pH 7.4 and 7.0 (Student's paired *t*-test). (B) Shows the normalised levels of mRNA for IκBα relative to β-actin. The mean  $\pm$  S.E. of three independent experiments is shown. # $P < 0.05$  for the difference between pH 7.4 and 7.0 (ANOVA).

protein. At pH 7.4, the mRNA levels increased within 10 min and continued to increase up to 30 min, as the NF-κB turned on the gene for its own inhibitor (Fig. 2B). There was also an increase in the mRNA for IκBα at pH 7.0 (which was somewhat greater than that at pH 7.4). The failure of IκBα protein to increase after its initial fall at pH 7.0 was therefore not due to a failure of its mRNA to increase and might indicate that the newly synthesised IκBα protein was degraded as rapidly as it was synthesised.

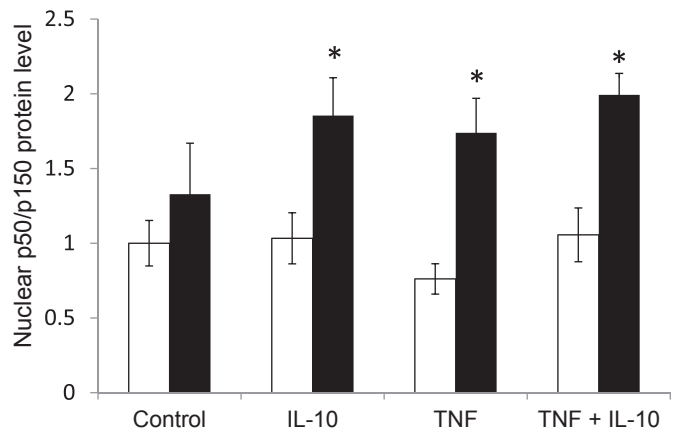
#### 3.4. Effects of low pH on p50/p105 levels

We used confocal microscopy to further probe the mechanisms involved by demonstrating that in the presence of either TNF or IL-10, or both together, there was an increase in the nuclear levels of p50/p105 (the antibody recognises both p50 and p105) in J774 cells at pH 7.0, but not at pH 7.4 (Fig. 3). There were no effects of pH on the nuclear levels of p50/p105 in the THP-1 cells (Fig. 3). These observations suggest that a low pH may increase the entry of p50 into the nuclei of J774 mouse macrophages and increase the binding of the inhibitory p50 homodimer to DNA, thus inhibiting cytokine secretion.

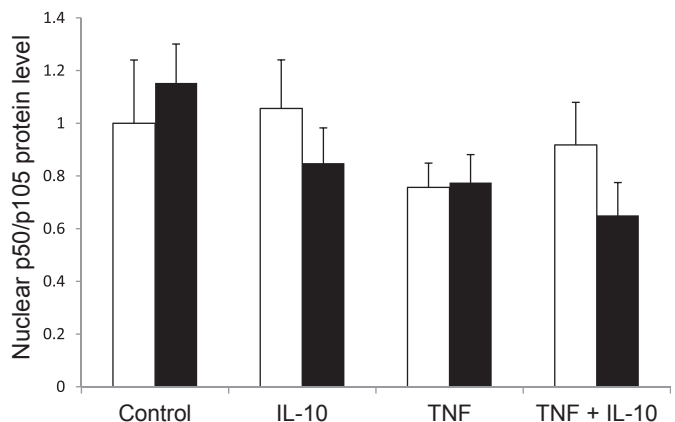
#### 3.5. Effects of low pH on NF-κB DNA-binding

We further investigated the effects of pH on NF-κB activity by assessing the binding of this transcription factor from nuclear extracts of mouse J774 macrophages to a consensus sequence oligonucleotide for the NF-κB promoter. Two specific bands were seen (Fig. 4A). The upper band corresponds to the binding of the p65:p50 heterodimer to the consensus DNA sequence and the lower band to the binding of the p50:p50 homodimer. A specific competitor (nonlabelled NF-κB consensus oligonucleotide) prevented the binding of the dimers to the radioactive oligonucleotide, but a nonspecific oligonucleotide (nonlabelled oct1 oligonucleotide) did not. Supershift analysis using an antibody to p50 retarded the mobility of both bands, whereas an antibody to p65 retarded the migration of only the top band, as expected. TNF stimulation of J774 mouse macrophages at pH 7.4 caused a transient increase in NF-κB p65:p50 DNA-binding, which peaked at 2 h (Fig. 4B). Reducing the extracellular pH to 7.0 led to an increased extent and prolongation

#### A: J774 macrophages

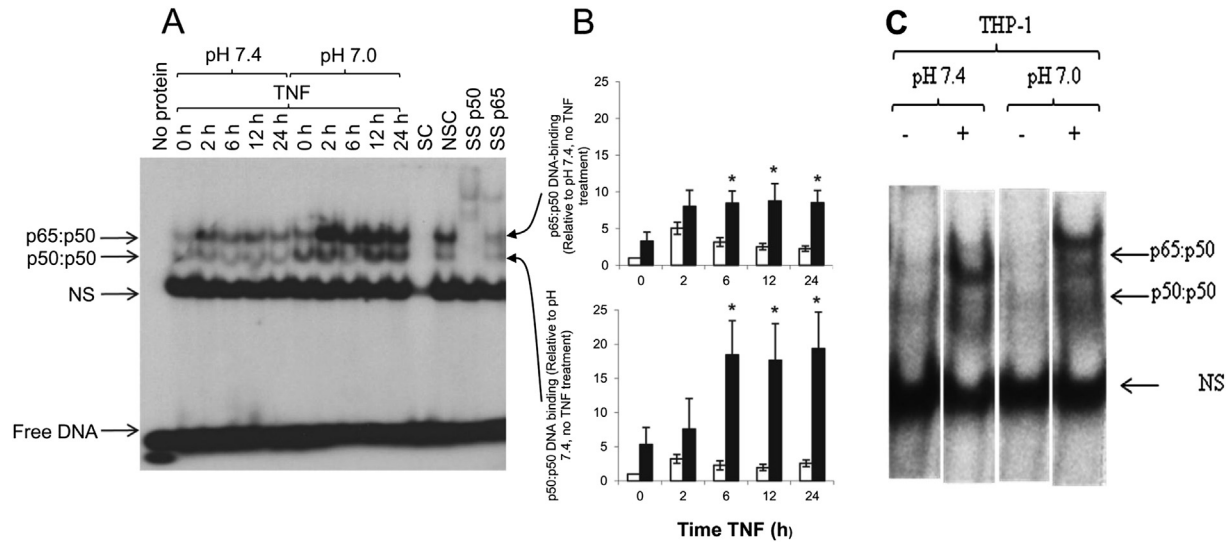


#### B: THP-1 macrophages



**Fig. 3.** Effects of pH on the levels of p50/p105 in macrophages. J774 (A) or THP-1 (B) cells were incubated for 24 h at 7.4 (white bars) or 7.0 (black bars) before treatment for 90 min with IL-10 (10 ng/ml), TNF (25 ng/ml), both together or in the absence of added cytokines (control). The nuclear levels of p50/p105 protein (the antibody recognises both p50 and p105), relative to the control at pH 7.4, were quantified by confocal microscopy. \* $P < 0.05$  compared to pH 7.4.



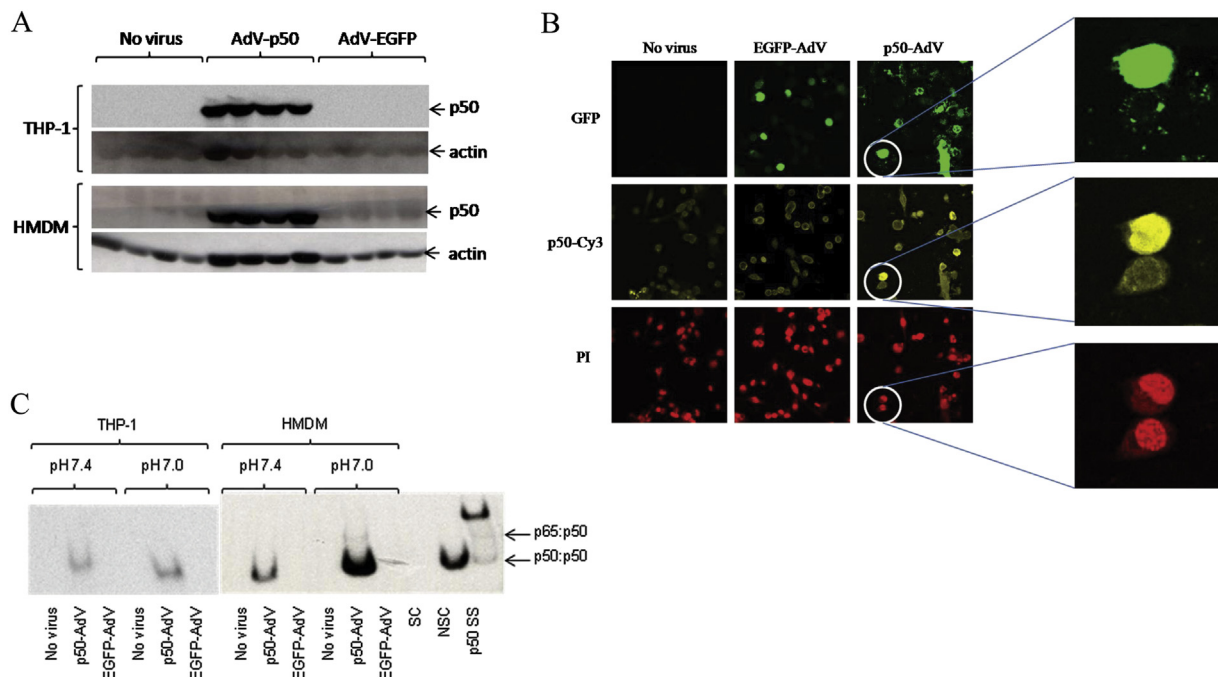


**Fig. 4.** Electrophoretic mobility shift assay of NF- $\kappa$ B binding to DNA to macrophages. (A) J774 cells were incubated for 24 h at 7.4 or 7.0 before treatment with TNF (25 ng/ml) for the indicated times. Nuclear extract was prepared and analysed for NF- $\kappa$ B binding by EMSA. Supershift analysis at 2 h at pH 7.4 using antibodies to p50 (SS p50) or p65 (SS p65) confirmed the presence of p50:p50 and p65:p50. SC: specific competitor (excess nonlabelled NF- $\kappa$ B consensus oligonucleotide). NSC: nonspecific competitor (excess nonlabelled nonrelated oligonucleotide). (B) The binding of p65:p50 was semi-quantified at pH 7.4 and (white bars) and 7.0 (black bars). The bars represent the mean  $\pm$  S.E. of three independent experiments. There was a significant difference between the binding at pH 7.4 and 7.0 ( $P < 0.05$ ; ANOVA) and between individual pH values ( $*P < 0.05$ ; Student's paired  $t$ -test). (C) THP-1 cells were incubated for 24 h at 7.4 or 7.0 before treatment with TNF (25 ng/ml) for 2 h. Nuclear extract was prepared and analysed for NF- $\kappa$ B binding by EMSA. The band above the p65:p50 band may possibly be p65:p65 homodimers. These results are representative of three independent experiments.

in p65:p50 DNA-binding for at least 24 h, which was even more marked for p50:p50 binding.

TNF increased NF- $\kappa$ B binding to DNA with the human THP-1 macrophages, but reducing the extracellular pH from 7.4 to 7.0

did not increase p65:p50 or p50:p50 DNA-binding (Fig. 4C), in contrast to the J774 mouse macrophages. A low extracellular pH may possibly have caused an increase in p65:p65 DNA-binding in the THP-1 cells (Fig. 4C).



**Fig. 5.** Effects of overexpression of p50. THP-1 human macrophages or HMDM were incubated with adenovirus containing p50 or EGFP. (A) Western blots showing that p50 is expressed in cells incubated with Adv-p50, but not in cells incubated with Adv-EGFP or without virus. Actin was used as a loading control. (B) Confocal microscopy showed that p50 (in yellow) was expressed in THP-1 macrophages following incubation with Adv-p50, but not with Adv-EGFP or without virus, even though both Adv-p50 and Adv-EGFP had been taken up by the cells (in green). The nuclei are shown by PI staining (in red). The insets show two cells (nuclei shown in red), the upper one of which had taken up Adv-p50 (green) but not the bottom one. The upper cell contains abundant p50 (yellow), whereas the lower cell does not. (C) THP-1 or human monocyte-derived macrophages were incubated for 24 h at 7.4 or 7.0. Nuclear extracts were prepared and analysed for NF- $\kappa$ B binding by EMSA. Supershift analysis of human monocyte-derived macrophages at pH 7.0 using an antibody to p50 (SS p50) confirmed the presence of much greater amounts of p50:p50 in the cells incubated with Adv-p50. SC: specific competitor (excess nonlabelled NF- $\kappa$ B consensus oligonucleotide). NSC: nonspecific competitor (excess nonlabelled nonrelated oligonucleotide). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.6. Effect of p50 overexpression on NF- $\kappa$ B activity

We investigated whether overexpressing p50 in THP-1 human macrophages or human-monocyte derived macrophages would confer the same pH sensitivity to NF- $\kappa$ B activity in human cells as was seen in mouse macrophages. Infection of THP-1 macrophages or HMDM with AdV-p50, but not AdV-EGFP, led to a marked increase in p50 protein expression (Fig. 5A). Confocal microscopy also showed an increase in expression of p50 in THP-1 cells incubated with AdV-p50 (Fig. 5B). Two cells are shown in a circle in Fig. 5B. The upper cell has been infected by the adenovirus, as shown by intense green fluorescence, whereas the lower cell was not been infected to any great extent. The upper cell shows intense fluorescence corresponding to p50 staining, whereas the bottom cell does not. Overexpression of p50 in THP-1 or human monocyte-derived macrophages led to an increase in constitutive p50 homodimer DNA-binding without increasing p65:p50 DNA-binding at pH 7.4 (Fig. 5C). Incubation of HMDM at pH 7.0 following AdV-p50 infection led to an increase in p50 homodimer DNA-binding, with little increase in p65:p50 DNA-binding.

Overexpression of p50 in THP-1 macrophages inhibited TNF secretion induced by LPS at 24 h by about 50% ( $P < 0.05$ ) and IL-6 secretion by about 85% ( $P < 0.05$ ) at pH 7.4 or 7.0 (Fig. 6). The control adenovirus had little or no effect (Fig. 6). Low extracellular pH (Fig. 6) or IL-10 (results not shown) had little effect on pro-inflammatory cytokine secretion. Similar results were obtained with 4 h incubation with LPS (results not shown). Fig. 6B demonstrates that reducing the pH from 7.4 to 7.0 did not affect TNF or IL-6 secretion by HMDM in response to LPS. AdV-p50, but not AdV-EGFP, had a tendency to inhibit LPS-induced TNF and IL-6 secretion in HMDM, but the inhibition was only modest.

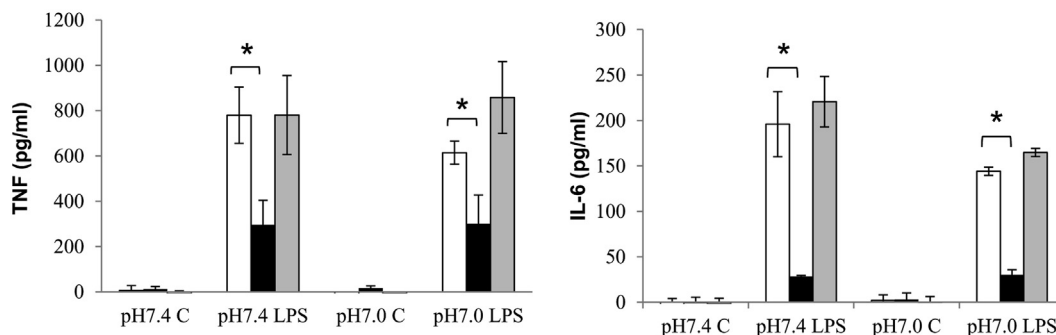
## 4. Discussion

Atherosclerotic lesions, as well as other sites of chronic inflammation, such as rheumatoid joints and tumours, have areas of low extracellular pH [3,4,6]. We observed that pro-inflammatory cytokine secretion stimulated by LPS was far less at pH 7.0 than at pH 7.4 for mouse J774 macrophages, but not for human monocyte-derived macrophages or human THP-1 macrophages (Figs. 1 and 6). It has been observed previously that J774 cells [41,42] and mouse peritoneal macrophages [43] secrete less TNF at low pH, but human monocyte-derived macrophages, THP-1 cells [44] and human monocytes [45] secrete more IL-1 $\beta$  at low pH. Our findings are in agreement with those of Grabowski et al. [42] using J774 macrophages, but extend their findings to look at the levels of I $\kappa$ B $\alpha$  over time and the binding of p65:p65 and p65:p50 dimers to the of NF- $\kappa$ B promoter.

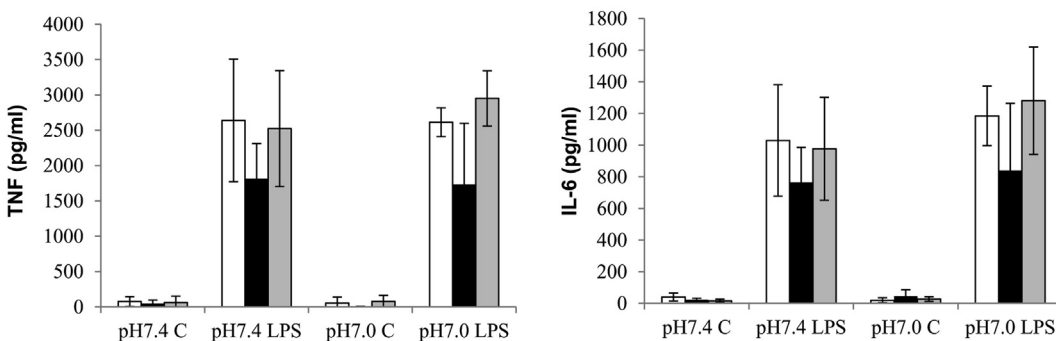
After stimulation, the levels of I $\kappa$ B $\alpha$  protein, the inhibitor of NF- $\kappa$ B, in the mouse macrophages decreased at both pH 7.4 and 7.0, as it was degraded, and returned back to normal within 1 h at pH 7.4, but remained depressed at pH 7.0 (Fig. 2A). The mouse macrophages increased their levels of mRNA for I $\kappa$ B $\alpha$  at both pH 7.4 and 7.0 (Fig. 2B). This observation would be consistent with the mRNA for I $\kappa$ B $\alpha$  being translated at pH 7.0, but the I $\kappa$ B $\alpha$  protein being continuously phosphorylated by IKK2, polyubiquitinated and degraded by proteasomes.

There was an increase in nuclear localisation of p50/p105 (the antibody we used recognised both proteins) in the mouse cells, but not in the human THP-1 cells, incubated with the pro-inflammatory cytokine TNF or the anti-inflammatory cytokine IL-10, or both together, at pH 7.0, but not at pH 7.4 (Fig. 3). IL-10 has been shown previously to increase selectively the nuclear localisation of p50

### A: THP-1 macrophages



### B: HMDM



**Fig. 6.** Effects of overexpression of p50 on cytokine secretion by macrophages. (A) THP-1 cells or (B) HMDM were infected with control adenovirus (grey bars) or adenovirus containing the gene for p50 (black bars) or left untreated (white bars). The cells were then incubated at pH 7.4 or 7.0 for 24 h and then with or without (C) LPS (100 pg/ml) for 24 h at these pH values. The medium was assayed for TNF or IL-6 by ELISA. \* $P < 0.05$ .



homodimers and this might help to explain why it is anti-inflammatory [37]. The reason why TNF also increased the level of p50 in the nucleus might have been because it induced the entry of pro-inflammatory p65:p50 heterodimers into the nucleus. There was a marked increase in the intensity and duration of NF- $\kappa$ B binding to a consensus oligonucleotide for the promoter of genes controlled by this transcription factor at low pH, especially for the p50:p50 homodimer (Fig. 4A and B), but no increase in p50:p50 homodimer or p65:p50 heterodimer binding at low pH in THP-1 macrophages (Fig. 4C). The p50:p50 homodimer has no trans-activation domain [46,47] and thus may block the activation of NF- $\kappa$ B by p65:p50 dimers. This may explain why the secretion of pro-inflammatory cytokines was reduced at low pH in the mouse cells. Bellocq et al. [48] noted the low pH induced an increase in p65:p50 DNA-binding and reporter gene activity in rat macrophages at 2 h, but did not comment on the increase in p50 homodimer DNA-binding. They showed an increase in TNF secretion at low pH, which is contrary to our results with mouse macrophages and other data with rabbit alveolar macrophages [49].

We overexpressed p50 in human macrophages to see if it would confer the same pH sensitivity to NF- $\kappa$ B activity in human cells as was present in mouse macrophages. Overexpression of p50 in human THP-1 macrophages using an adenovirus inhibited pro-inflammatory cytokine secretion considerably (Fig. 6A). We observed that lower transfection efficiencies, using lipid-mediated or electroporation techniques, had varied and often opposing effects on the cytokine secretion profile of the macrophages (Gerry and Leake, unpublished observations). p50 has a higher affinity for p65 than itself [50] and so it may be necessary to increase p50 levels substantially to increase p50:p50 homodimer formation. Overexpression of p50 in human THP-1 or HMDM did not, however, lead to the appearance of an inhibitory effect of pH on cytokine secretion at low pH (Fig. 6). Lowering the pH from 7.4 to 7.0, in either the presence or absence of p50 overexpression, had no effect on cytokine secretion in the human macrophages, in contrast to the mouse macrophages (Fig. 1).

A low extracellular pH within atherosclerotic lesions might possibly have different effects in mice and humans, increasing the activity of p50:p50 homodimers in mice but not in humans. This might decrease the expression of pro-inflammatory cytokines in mice and may be one factor to help explain why atherosclerosis is less severe in mice (unless genetically modified) than in humans.

IL-10 is produced by many cell types and is generally considered to be anti-inflammatory [34,51], as it suppresses the production of inflammatory cytokines, such as IL-6 and TNF. IL-10 has been shown to block nuclear translocation of p65, but to induce nuclear translocation of p50 and to increase the binding of p50:p50 homodimers to DNA [37]. We therefore tested the effects of IL-10 on pro-inflammatory cytokine secretion. Interestingly, IL-10 and low pH inhibited pro-inflammatory cytokine secretion in mouse J774 macrophages, but neither inhibited pro-inflammatory cytokine secretion in human THP-1 macrophages, regardless of whether or not p50 had been overexpressed in the THP-1 cells (Fig. 1 and results not shown). The reason for the different behaviour of the IL-10 in the two types of macrophages is unknown.

A moderate reduction of pH greatly decreased inflammatory cytokine secretion in mouse, but not in human macrophages. The DNA-binding activity of NF- $\kappa$ B, especially for p50:p50 homodimers, was greatly increased and prolonged at low pH in the mouse macrophages. The effects of pH on inflammation in atherosclerosis are complex because, as well as regulating inflammatory genes, NF- $\kappa$ B is involved in the resolution of inflammation [52,53].

## Funding

This work was supported by a BBSRC research studentship (ABG) and a British Heart Foundation Project Grant (Number PG/04/015/16658 to D.S.L.).

## Acknowledgements

We thank Dr Katrina Bicknell (Pharmacy, University of Reading) for advice and the EGFP virus and Dr Nikki Horwood (Imperial College London) for the p50 virus.

## References

- [1] Libby P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2012;32:2045–51.
- [2] Davies MJ, Thomas A. Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. *N Engl J Med* 1984;310:1137–40.
- [3] Farr M, Garvey K, Bold AM, Kendall MJ, Bacon PA. Significance of the hydrogen ion concentration in synovial fluid in rheumatoid arthritis. *Clin Exp Rheumatol* 1985;3:99–104.
- [4] Griffiths JR. Are cancer cells acidic? *Br J Cancer* 1991;64:425–7.
- [5] Leake DS. Does an acidic pH explain why low density lipoprotein is oxidised in atherosclerotic lesions? *Atherosclerosis* 1997;129:149–57.
- [6] Naghavi M, John R, Naguib S, et al. pH heterogeneity of human and rabbit atherosclerotic plaques; a new insight into detection of vulnerable plaque. *Atherosclerosis* 2002;164:27–35.
- [7] Khan T, Soller B, Naghavi M, Casscells W. Tissue pH determination for the detection of metabolically active, inflamed vulnerable plaques using near-infrared spectroscopy: an in vitro feasibility study. *Cardiology* 2005;103:10–6.
- [8] Morgan J, Leake DS. Acidic pH increases the oxidation of LDL by macrophages. *FEBS Lett* 1993;333:275–9.
- [9] Patterson RA, Leake DS. Human serum, cysteine and histidine inhibit the oxidation of low density lipoprotein less at acidic pH. *FEBS Lett* 1998;434:317–21.
- [10] Gerry AB, Leake DS. A moderate reduction in extracellular pH protects macrophages against apoptosis induced by oxidized low density lipoprotein. *J Lipid Res* 2008;49:782–9.
- [11] Lee-Rueckert M, Lappalainen J, Leinonen H, Pihlajamäki T, Jauhainen M, Kovanen PT. Acidic extracellular environments strongly impair ABCA1-mediated cholesterol efflux from human macrophage foam cells. *Arterioscler Thromb Vasc Biol* 2010;30:1766–72.
- [12] Amann K, Tyralla K, Gross ML, Eifert T, Adamczak M, Ritz E. Special characteristics of atherosclerosis in chronic renal failure. *Clin Nephrol* 2003;60:S13–21.
- [13] Makarov SS. NF- $\kappa$ B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res* 2001;3:200–6.
- [14] Fantini MC, Pallone F. Cytokines: from gut inflammation to colorectal cancer. *Curr Drug Targets* 2008;9:375–80.
- [15] Robert C, Kupper TS. Mechanisms of disease: inflammatory skin diseases, T cells, and immune surveillance. *N Engl J Med* 1999;341:1817–28.
- [16] Brand K, Page S, Rogler G, et al. Activated transcription factor nuclear factor- $\kappa$ B is present in the atherosclerotic lesion. *J Clin Invest* 1996;97:1715–22.
- [17] Hayden MS, Ghosh S. NF- $\kappa$ B, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* 2012;26:203–34.
- [18] May MJ, Ghosh S. Rel/NF- $\kappa$ B and I $\kappa$ B proteins: an overview. *Semin Cancer Biol* 1997;8:63–73.
- [19] Fujita T, Nolan GP, Ghosh S, Baltimore D. Independent modes of transcriptional activation by the p50-subunit and p65-subunit of NF- $\kappa$ B. *Genes Dev* 1992;6:775–87.
- [20] Ghosh S, May MJ, Kopp EB. NF- $\kappa$ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998;16:225–60.
- [21] Fujita T, Nolan GP, Liou HC, Scott ML, Baltimore D. The candidate proto-oncogene Bcl-3 encodes a transcriptional coactivator that activates through NF- $\kappa$ B p50 homodimers. *Genes Dev* 1993;7:1354–63.
- [22] Monaco C, Paleolog E. Nuclear factor kappa B: a potential therapeutic target in atherosclerosis and thrombosis. *Cardiovasc Res* 2004;61:671–82.
- [23] de Winther MPJ, Kanters E, Kraal G, Hofker MH. Nuclear factor  $\kappa$ B signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* 2005;25:904–14.
- [24] Van der Heiden K, Cuhlmann S, Luong LA, Zakkar M, Evans PC. Role of nuclear factor  $\kappa$ B in cardiovascular health and disease. *Clin Sci* 2010;118:593–605.
- [25] Kanters E, Pasparakis M, Gijbels MJJ, et al. Inhibition of NF- $\kappa$ B activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2003;112:1176–85.
- [26] Park SH, Sui YP, Gizard F, et al. Myeloid-specific I $\kappa$ B kinase  $\beta$  deficiency decreases atherosclerosis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2012;32:2869–76.
- [27] Yoshimura S, Morishita R, Hayashi K, et al. Inhibition of intimal hyperplasia after balloon injury in rat carotid artery model using cis-element 'decoy' of

- nuclear factor- $\kappa$ B binding site as a novel molecular strategy. *Gene Ther* 2001;8:1635–42.
- [28] Cao CC, Zhu YF, Chen W, et al. IKK $\epsilon$  knockout prevents high fat diet induced arterial atherosclerosis and NF- $\kappa$ B signaling in mice. *Plos One* 2013;8.
- [29] Mallavia B, Recio C, Oguiza A, et al. Peptide inhibitor of NF- $\kappa$ B translocation ameliorates experimental atherosclerosis. *Am J Pathol* 2013;182:1910–21.
- [30] Chiba T, Kondo Y, Shinozaki S, et al. A selective NF $\kappa$ B inhibitor, DHMEQ, reduced atherosclerosis in ApoE-deficient mice. *J Atheroscler Thromb* 2006;13:308–13.
- [31] Gareus R, Kotsaki E, Xanthoulea S, et al. Endothelial cell-specific NF- $\kappa$ B inhibition protects mice from atherosclerosis. *Cell Metab* 2008;8:372–83.
- [32] Kanter E, Gijbels MJJ, van der Made I, et al. Hematopoietic NF- $\kappa$ B1 deficiency results in small atherosclerotic lesions with an inflammatory phenotype. *Blood* 2004;103:934–40.
- [33] Moore KW, Malefyt RD, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765.
- [34] Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy – review of a new approach. *Pharmacol Rev* 2003;55:241–69.
- [35] Schottelius AJG, Mayo MW, Sartor RB, Badwin AS. Interleukin-10 signaling blocks inhibitor of kappa B kinase activity and nuclear factor kappa B DNA binding. *J Biol Chem* 1999;274:31868–74.
- [36] Kuwata H, Watanabe Y, Miyoshi H, et al. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF- $\alpha$  production in macrophages. *Blood* 2003;102:4123–9.
- [37] Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ. Molecular mechanisms of interleukin-10-mediated inhibition of NF- $\kappa$ B activity: a role for p50. *Clin Exp Immunol* 2004;135:64–73.
- [38] Firth CA, Yang YT, Gieseg SP. Lipid oxidation predominates over protein hydroperoxide formation in human monocyte-derived macrophages exposed to aqueous peroxy radicals. *Free Radic Res* 2007;41:839–48.
- [39] Harris LK, Mann GE, Ruiz E, Mushtaq S, Leake DS. Ascorbate does not protect macrophages against apoptosis induced by oxidised low density lipoprotein. *Arch Biochem Biophys* 2006;455:68–76.
- [40] Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [41] Heming TA, Tuazon DM, Dave SK, Chopra AK, Peterson JW, Bidani A. Post-transcriptional effects of extracellular pH on tumour necrosis factor- $\alpha$  production in RAW 246.7 and J774 A.1 cells. *Clin Sci* 2001;100:259–66.
- [42] Grabowski J, Vazquez DE, Costantini T, et al. Tumor necrosis factor expression is ameliorated after exposure to an acidic environment. *J Surg Res* 2012;173:127–34.
- [43] Mogi C, Tobo M, Tomura H, et al. Involvement of proton-sensing TDAG8 in extracellular acidification-induced inhibition of proinflammatory cytokine production in peritoneal macrophages. *J Immunol* 2009;182:3243–51.
- [44] Rajamäki K, Nordstrom T, Nurmi K, et al. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. *J Biol Chem* 2013;288:13410–9.
- [45] Jancic CC, Cabrini M, Gabelloni ML, et al. Low extracellular pH stimulates the production of IL-1 beta by human monocytes. *Cytokine* 2012;57:258–68.
- [46] Baeuerle PA, Henkel T. Function and activation of NF- $\kappa$ B in the immune system. *Annu Rev Immunol* 1994;12:141–79.
- [47] Hayden MS, Ghosh S. Signaling to NF- $\kappa$ B. *Genes Dev* 2004;18:2195–224.
- [48] Bellocq A, Suberville S, Philippe C, et al. Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages – evidence for involvement of nuclear factor- $\kappa$ B activation. *J Biol Chem* 1998;273:5086–92.
- [49] Bidani A, Wang CZ, Saggi SJ, Heming TA. Evidence for pH sensitivity of tumor necrosis factor- $\alpha$  release by alveolar macrophages. *Lung* 1998;176:111–21.
- [50] Chen FE, Huang D-B, Chen Y-Q, Ghosh G. Crystal structure of p50/p65 heterodimer of transcription factor NF- $\kappa$ B bound to DNA. *Nature* 1998;391:410–3.
- [51] Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011;473:317–25.
- [52] Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF- $\kappa$ B in the resolution of inflammation. *Nat Med* 2001;7:1291–7.
- [53] Lawrence T, Bebiën M, Liu GY, Nizet V, Karin M. IKK $\alpha$  limits macrophage NF- $\kappa$ B activation and contributes to the resolution of inflammation. *Nature* 2005;434:1138–43.