Provided for non-commercial research and educational use only.
Not for reproduction or distribution or commercial use.

This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator.

All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

http://www.elsevier.com/locate/permissionusematerial

ELSEVIER

Journal of Cystic Fibrosis 6 (2007) 304–308

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Early inflammation in the airways of a cystic fibrosis foetus ☆

Catherine Verhaeghe [a], Katty Delbecque [b], Laurence de Leval [b], Cecile Oury [a], Vincent Bours [a,*]

[a] *Department of Human Genetics, Center for Biomedical Integrative Genoproteomics (CBIG), University of Liege, B-4000 Liege, Belgium*
[b] *Department of Pathology, Center for Biomedical Integrative Genoproteomics (CBIG), University of Liege, B-4000 Liege, Belgium*

Received 12 October 2006; received in revised form 22 November 2006; accepted 7 December 2006
Available online 12 January 2007

Abstract

In cystic fibrosis patients, inflammation is often considered to be secondary to chronic infections. In the present study, we show increased levels of pro-inflammatory proteins in the lungs of a cystic fibrosis foetus compared to the lungs of two normal foetuses. Our findings suggest therefore the existence of an early intrinsic pro-inflammatory state in cystic fibrosis airways.
© 2006 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: CFTR; NF-κB; Pro-inflammatory cytokines

1. Introduction

Eighty to ninety percents of Cystic Fibrosis (CF) patients succumb to respiratory failure caused by chronic bacterial infection and concomitant airway inflammation [2]. The sequence of events at the onset of pulmonary infection and inflammation is controversial and not fully characterized. At birth, the lungs of CF patients appear normal [3,4]; however, autopsy examination of infants revealed a dilatation of acinar and duct lumens of sub-mucosal glands due to abnormal mucus secretion demonstrating pre-clinical signs in the very first months of life. Several studies also described the presence of neutrophils, elevated levels of elastase and IL-8, in the absence of any pathogen, in broncho-alveolar lavage fluids of CF newborns as compared to healthy individuals, opening the debate on the origin of this inflammation [2,5–7]. Indeed, the abnormal composition of airway secretions and/or their depletion are frequently cited as the host factors that predisposes CF patients to chronic colonization by *P. aeruginosa* and resultant inflammation. However, a recent study [8] showed that the over-expression of the epithelial Na^+ channel ENac in mice bronchiolar epithelium reduced the volume of preciliary liquid leading to neutrophil influx and increased levels of IL-8 in airways. These results indicate that inflammation can arise from disregulated ion transport in airway epithelium in the absence of any infection. Moreover, several reports showed in various CF cell lines [9–12] an increased activation of the transcription factor NF-κB, a central player in inflammation, supporting the idea that CFTR dysfunction can by itself cause the expression of pro-inflammatory mediators [2,5–7,13–15]. Nevertheless, very little evidence for an inflammatory state existing before infection have been brought so far, leaving the debate open.

2. Case report

The examination of a 24 week-old foetus after pregnancy interruption for a hydrocephaly of unknown origin showed pancreatic histology signs evoking CF. There was not any sign of infection in the mother or the foetus. A genetic test highlighted the ΔF508 mutation on both CFTR alleles and

☆ C. V. was supported by a F. R. I. A. ("Fond pour la Recherche Industrielle et Agricole") fellowship. C. O. and L. de L. are Research Associates at the National Fund for Scientific Research (FNRS, Belgium). This work was supported by grants from National Fund for Scientific Research and Foundation Forton of Belgium. Some data have been previously reported in the form of an abstract (international meeting "Cystic Fibrosis and Anionic Channelopathies", Brussels, September 8, 2006 and the 28th European CF Conference, June 22–25, 2005, Crete, Greece [1]).
* Corresponding author. Department of Human Genetics, University of Liège, CHU Sart-Tilman B35, B-4000 Liège, Belgium. Tel.: +32 4 366 81 44; fax: +32 4 366 81 46.
E-mail address: vbours@ulg.ac.be (V. Bours).

1569-1993/$ - see front matter © 2006 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2006.12.001

Author's personal copy

thus confirmed the CF diagnosis. To date, no relation has been established between hydrocephaly and cystic fibrosis.

Two foetuses were obtained after termination of the pregnancies either for a CMV foetopathy (22 weeks of amenorrhea) or for a Turner syndrome (23 weeks of amenorrhea) and were used as controls. Lungs were collected after the approval from the Institutional Review Board.

3. Methods

3.1. Immunohistochemistry and immunofluorescence

Studies were performed on paraffin-embedded material. Primary antibodies used were anti-ICAM-1, anti-Gro-β, anti-Gro-γ, anti-p65 (Santa-Cruz), anti-MMP1 (R and D system, France) and anti-COX2 (Cayman, MI).

Immunohistochemistry. A three-step indirect immunoperoxidase technique was used with the LSAB2 kit and DAB+(Dako) as the chromogen after heat-induced antigen retrieval in EDTA buffer (pH 9).

Immunofluorescence. After heat-induced antigen retrieval in sodium citrate buffer (10 mM, pH 6), sections were stained with primary antibody followed by incubation with Alexa-Fluor 488 (Molecular Probes, Leiden, The Netherlands). For nuclear DNA staining, TOTO-3 iodide (Molecular Probes) was added to the secondary antibody solution. Confocal microscopy analyses were performed with a TCS SP confocal microscope (Leica) as described [16].

4. Results and discussion

It is debated whether pulmonary inflammation precedes or follows infection in CF. The lungs of patients with CF are inflamed and infected at a young age, and it is difficult to exclude the presence of all pathogens. Therefore, to investigate a putative early pro-inflammatory state, immunohistological studies were undertaken to compare the expression levels of known pro-inflammatory proteins in the lungs of a 24 week-old CF foetus homozygous for the ΔF508-CFTR mutation and in control lungs from two foetuses at similar development stages. Due to scarcity of such human material, this is the first study of CF foetal lungs.

Since an early pathological hallmark of CF inflammatory response consists of neutrophil influx into the airways, we analysed the expression of the intracellular adhesion molecule-1 (ICAM-1) and the chemokines Gro-β and Gro-γ, three pro-inflammatory molecules known to be involved in neutrophil extravasation and chemotaxis [17,18]. We also studied the expression of the major pro-inflammatory enzyme Cox-2 and of the matrix metalloproteinase 1 (MMP-1) that by degrading extracellular matrix proteins may play a role in airway remodeling.

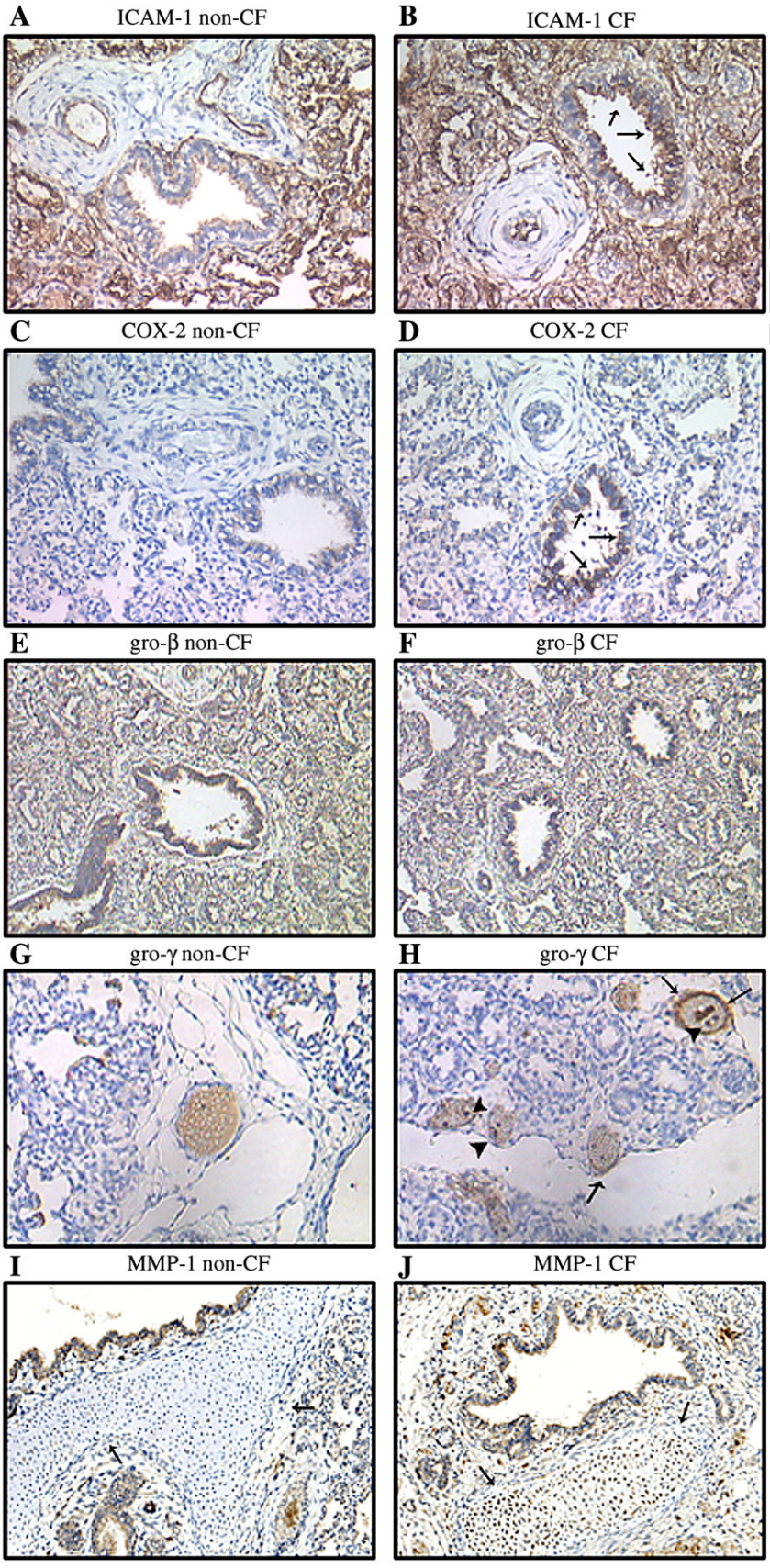
ICAM-1 expression was higher in CF bronchial epithelial cells as compared to control non-CF foetal lung epithelia; ICAM-1 was mainly localized at the apical membrane of these bronchial cells (Fig. 1A and B). High ICAM-1 protein levels were previously observed in adult CF lungs, small intestines and proliferating bile-ducts [19–21]. Increased Gro-γ expression was observed in CF foetal lungs, specifically in monocytes and endothelial cells (Fig. 1G and H). In contrast, Gro-β expression was identical in CF and control foetal lungs (Fig. 1E and F). The role of monocytes in CF inflammation has been suggested by Zaman et al. [22] who showed that basal and LPS-induced IL-8 expression by CF monocytes is higher than IL-8 secretion by normal monocytes.

The enzyme Cox-2 plays a central role in inflammation [23–25]. Its over-expression was observed in bronchiolar epithelial cells from CF lungs in comparison with development-matched control lungs (Fig. 1C and D). MMP-1 was found to be expressed in the CF pulmonary cartilage while it was not detected in the cartilage of control foetuses (Fig. 1I and J). Although an over-expression of MMP-1 has never been described in CF patients, increased levels of this matrix metalloproteinase is associated with several human diseases such as cancer, autoimmune disorders, COPD and asthma [26–28]. Moreover, an increased MMP-1 expression has also been shown in cartilage tissue of patients with osteoarthritis [29].

Interestingly, despite of the absence of any histological sign of inflammation, the CF foetal lungs thus over-expressed MMP-1, Groγ, ICAM-1 and Cox-2, four molecules known to be regulated by NF-κB [30–33]. NF-κB is an inducible transcription factor that plays a central role in the regulation of immune and inflammatory responses. While NF-κB is required for cell survival and immunity, prolonged NF-κB activation seems essential for the persistence of chronic inflammatory diseases [34–36]. Constitutive activation of this inflammatory transcription factor has been already associated with CF [10–12]. We therefore determined whether the CF foetal lungs displayed higher NF-κB activation than control lungs. For this purpose, subcellular immunolocalization analyses of p65 were performed (Fig. 2A and B); these analyses revealed a cytoplasmic localization of p65 both in CF (Fig. 2B) and non-CF (Fig. 2A) lung cells. A nuclear staining of p65 was only observed in the CF lung epithelial cells (Fig. 2B). This result indicated that NF-κB is activated in the CF foetal lungs. Our findings therefore suggest that, already in CF foetal lungs, CFTR dysfunction leads to NF-κB activation which enhances the expression of specific pro-inflammatory proteins.

To our knowledge, this is the first report of *in vivo* evidence for a pro-inflammatory process initiated very early in the pathogenesis of cystic fibrosis before any infection. This intrinsic inflammation is probably mild and likely results from a deregulated NF-κB activation which may be the starting point for subsequent pathological pulmonary dysfunction. The identification of the molecular mechanisms linking mutated CFTR to the NF-κB activation pathway will be crucial to define novel anti-inflammatory strategies for CF patients. Indeed, as inflammation and neutrophil influx are two major pathophysiological features of pulmonary CF disease, any therapy that reduces the production of pro-

Author's personal copy


A
ICAM-1 non-CF
B
ICAM-1 CF
C
COX-2 non-CF
D
COX-2 CF
E
gro-β non-CF
F
gro-β CF
G
gro-γ non-CF
H
gro-γ CF
I
MMP-1 non-CF
J
MMP-1 CF

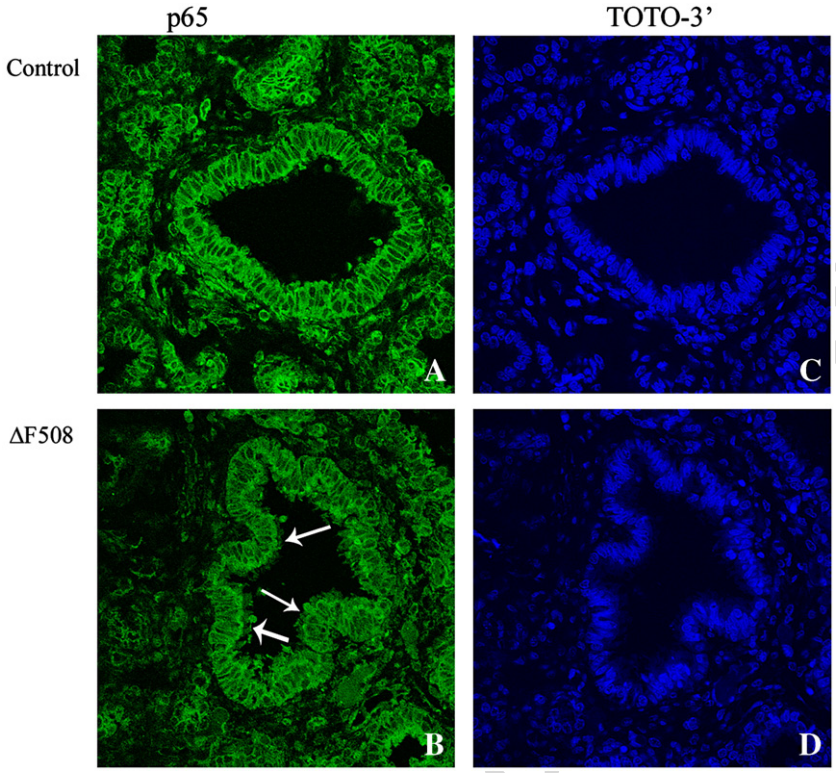


Fig. 2. Nuclear localization of p65 in the CF foetal lung. Control foetal lungs (A) and ΔF508-CFTR foetal lungs (B) were stained for p65 (A, B) localization. Cell nuclear DNA was stained with TOTO-3 (C–D). Original magnification, ×63.

inflammatory molecules could, if applied early, prevent the chronic inflammation and the degradation of lung function. Many NF-κB inhibitors are currently being developed and tested in various conditions. Our observations and previous reports provide a rationale for further studies on *in vivo* NF-κB activity before infection in CF lungs and possibly for clinical trials with specific and tolerable NF-κB inhibitors.

Acknowledgments

We thank Leon-Marie Dupuis for his excellent technical assistance.

References

[1] Abstracts of the 28th European Cystic Fibrosis Conference, Hersonissos, Crete, Greece, 22–25 June 2005. J Cyst Fibros 2005; 4 Suppl 1: S1-143, SI-IX.
[2] Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 2002;15:194–222.
[3] Chow CW, Landau LI, Taussig LM. Bronchial mucous glands in the newborn with cystic fibrosis. Eur J Pediatr 1982;139:240–3.
[4] Ornoy A, Arnon J, Katznelson D, Granat M, Caspi B, Chemke J. Pathological confirmation of cystic fibrosis in the fetus following prenatal diagnosis. Am J Med Genet 1987;28:935–47.
[5] Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. J Allergy Clin Immunol 1999;104:72–8.
[6] Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW. Early pulmonary inflammation in infants with cystic fibrosis. Am J Respir Crit Care Med 1995;151:1075–82.
[7] Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. J Infect Dis 1997;175:638–47.
[8] Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med 2004;10:487–93.
[9] Knorre A, Wagner M, Schaefer HE, Colledge WH, Pahl HL. DeltaF508-CFTR causes constitutive NF-kappaB activation through an ER-overload response in cystic fibrosis lungs. Biol Chem 2002;383:271–82.
[10] Venkatakrishnan A, Stecenko AA, King G, Blackwell TR, Brigham KL, Christman JW, et al. Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. Am J Respir Cell Mol Biol 2000;23:396–403.
[11] Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, et al. Relationship between IkappaBalpha deficiency, NFkappaB activity and interleukin-8 production in CF human airway epithelial cells. Pflugers Arch 2001;443(Suppl 1):S40–4.

Fig. 1. Over-expression of pro-inflammatory proteins in a CF foetal lung. Control (A, C, E, G, I) and CF (B, D, F, H, J) foetal lungs were stained for ICAM-1 (A, B), COX-2 (C, D), Gro-β (E, F), Gro-γ (G, H) and MMP-1 (I, J) expressions. Arrows indicate over-expressed pro-inflammatory molecules in bronchiolar epithelial cells (A–D), in endothelial cells (G, H) or in cartilage (I, J). Arrowheads indicate Groγ expression in monocytes. Original magnification, ×200 (A–F, I–J), ×400 (G, H).

Author's personal copy

[12] DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 1997;388:548–54.
[13] Armstrong DS, Hook SM, Jamsen KM, Nixon GM, Carzino R, Carlin JB, et al. Lower airway inflammation in infants with cystic fibrosis detected by newborn screening. Pediatr Pulmonol 2005;40:500–10.
[14] Dakin CJ, Numa AH, Wang H, Morton JR, Vertzyas CC, Henry RL. Inflammation, infection, and pulmonary function in infants and young children with cystic fibrosis. Am J Respir Crit Care Med 2002;165: 904–10.
[15] Delacourt C. Inflammation and cystic fibrosis. Arch Pediatr 2003;10 (Suppl 2):338s–41s.
[16] Gillet L, Daix V, Donofrio G, Wagner M, Koszinowski UH, China B, et al. Development of bovine herpes virus 4 as an expression vector using bacterial artificial chromosome cloning. J Gen Virol 2005;86:907–17.
[17] Rollins BJ. Chemokines. Blood 1997;90:909–28.
[18] Guo RF, Ward PA. Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. Free Radic Biol Med 2002;33:303–10.
[19] Kinnman N, Lindblad A, Housset C, Buentke E, Scheynius A, Strandvik B, et al. Expression of cystic fibrosis transmembrane conductance regulator in liver tissue from patients with cystic fibrosis. Hepatology 2000;32:334–40.
[20] Raia V, Maiuri L, de Ritis G, de Vizia B, Vacca L, Conte R, et al. Evidence of chronic inflammation in morphologically normal small intestine of cystic fibrosis patients. Pediatr Res 2000;47:344–50.
[21] Hubeau C, Lorenzato M, Couetil JP, Hubert D, Dusser D, Puchelle E, et al. Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa. Clin Exp Immunol 2001;124:69–76.
[22] Zaman MM, Gelrud A, Junaidi O, Regan MM, Warny M, Shea JC, et al. Interleukin 8 secretion from monocytes of subjects heterozygous for the deltaF508 cystic fibrosis transmembrane conductance regulator gene mutation is altered. Clin Diagn Lab Immunol 2004;11:819–24.
[23] Park GY, Christman JW. Involvement of cyclooxygenase-2 and prostaglandins in the molecular pathogenesis of inflammatory lung diseases. Am J Physiol Lung Cell Mol Physiol 2006;290:L797–805.
[24] Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, et al. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. FASEB J 2004;18:300–10.
[25] Cox DG, Crusius JB, Peeters PH, Bueno-de-Mesquita HB, Pena AS, Canzian F. Haplotype of prostaglandin synthase 2/cyclooxygenase 2 is involved in the susceptibility to inflammatory bowel disease. World J Gastroenterol 2005;11:6003–8.
[26] Rajah R, Nachajon RV, Collins MH, Hakonarson H, Grunstein MM, Cohen P. Elevated levels of the IGF-binding protein protease MMP-1 in asthmatic airway smooth muscle. Am J Respir Cell Mol Biol 1999;20:199–208.
[27] Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, et al. Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. Am J Respir Crit Care Med 2001;163:786–91.
[28] Pardo A, Selman M. MMP-1: the elder of the family. Int J Biochem Cell Biol 2005;37:283–8.
[29] Ray A, Kuroki K, Cook JL, Bal BS, Kenter K, Aust G, et al. Induction of matrix metalloproteinase 1 gene expression is regulated by inflammation-responsive transcription factor SAF-1 in osteoarthritis. Arthritis Rheum 2003;48:134–45.
[30] Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J Biol Chem 1995;270:31315–20.
[31] Vincenti MP, Coon CI, Brinckerhoff CE. Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts. Arthritis Rheum 1998;41:1987–94.
[32] Tian B, Nowak DE, Jamaluddin M, Wang S, Brasier AR. Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signaling. J Biol Chem 2005;280:17435–48.
[33] van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, et al. 12-*O*-tetradecanoylphorbol-13-acetate-and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. J Biol Chem 1994;269:6185–92.
[34] Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol 2002;2:725–34.
[35] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci 2005;30:43–52.
[36] Giuliani C, Napolitano G, Bucci I, Montani V, Monaco F. Nf-kB transcription factor: role in the pathogenesis of inflammatory, autoimmune, and neoplastic diseases and therapy implications. Clin Ter 2001;152:249–53.

Author's personal copy