

## Interleukin-32 $\alpha$ expression in human colonic subepithelial myofibroblasts

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**Abstract.** Interleukin (IL)-32 is a recently described proinflammatory cytokine, characterized by induction of nuclear factor (NF)- $\kappa$ B activation. We studied IL-32 $\alpha$  expression in human colonic subepithelial myofibroblasts (SEMFs). Colonic SEMFs were isolated from normal human colon tissue. IL-32 $\alpha$  protein expression was evaluated by Western blot analyses, and IL-32 $\alpha$  mRNA expression was analyzed by real-time PCR. IL-32 $\alpha$  mRNA was weakly expressed without a stimulus, and its expression was markedly enhanced by IL-1 $\beta$  and TNF- $\alpha$ . IL-1 $\beta$  and TNF- $\alpha$  enhanced intracellular accumulation of IL-32 $\alpha$  protein, but IL-32 $\alpha$  was not detected in supernatants. Each cytokine dose- and time-dependently induced IL-32 $\alpha$  mRNA expression. An inhibitor of phosphatidylinositol 3-kinase (LY294002) significantly suppressed IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blotting. Blockade of NF- $\kappa$ B activation by an adenovirus expressing a stable mutant form of I $\kappa$ B $\alpha$  markedly suppressed IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression. Human colonic SEMFs expressed IL-32 $\alpha$  in response to IL-1 $\beta$  and TNF- $\alpha$ . IL-32 $\alpha$  mRNA expression depends on the phosphatidylinositol 3-kinase and the NF- $\kappa$ B system.

### Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastro-

intestinal tract. IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora (1-3). This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system. The investigation of the immunological responses in the intestinal mucosa is a fundamental approach to the understanding of the pathophysiology of IBD.

Interleukin (IL)-32 is a recently described cytokine that is produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells (4,5). Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that epithelial cells are the dominant and widespread source (6). The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons (7). There are four splice variants (IL-32 $\alpha$ , IL-32 $\beta$ , IL-32 $\delta$  and IL-32 $\gamma$ ), and IL-32 $\alpha$  is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)- $\gamma$  in lung epithelial cells and monocytes (4). IL-32 exhibits several properties typical of proinflammatory cytokines (4,5). For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8 by means of the activation of NF- $\kappa$ B and p38 mitogen-activated protein kinases (MAPKs) (4,5).

Netea *et al* recently demonstrated that IL-32 augments the production of IL-1 $\beta$  and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism (5). NODs are a family of intracytoplasmic bacterial sensors, and recognition of bacterial peptidoglycans subsequently induces NF- $\kappa$ B activation (8). Mutations in *NOD2* have been implicated in the pathogenesis of CD (9,10), and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in cytokine production (11,12). Recently, it has been shown that the *NOD2* mutation in CD patients potentiates NF- $\kappa$ B activity and IL-1 $\beta$  processing (13). Thus, these data suggest a pivotal role of IL-32 in the pathophysiology of IBD and in particular of CD.

In this study, we investigated the expression of IL-32 $\alpha$  in human colonic subepithelial myofibroblasts (SEMFs). Colonic SEMFs are  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)-positive

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mesenchymal cells and are located subjacent to the basement membrane of the small and large intestines. These cells belong to a family of  $\alpha$ -SMA positive fibroblast-like cells, such as the cells of the hepatic stellate (Ito) renal mesangial/tubulointerstitial, the lung interstitial contractile cells, and the pancreatic stellate cells (14-16). Colonic SEMFs act to mediate information flow in both directions to and from the intestinal epithelium and the immune and the other mesenchymal and neural elements of the lamina propria, and play an important role in ECM metabolism affecting the growth of the basement membrane in the intestinal mucosa. Current observations suggest a role of colonic SEMFs in the pathophysiology of IBD via IL-32 expression.

## Materials and methods

**Reagents.** Recombinant human IL-1 $\beta$ , IL-17 and IFN- $\gamma$  were purchased from R&D Systems (Minneapolis, MN), and other cytokines were obtained from PeproTech (Rocky Hill, NJ). Anti-human IL-32 $\alpha$  antibodies were purchased from R & D Systems. All other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

**Culturing of human colonic SEMFs and colonic epithelial cell lines.** Primary colonic SEMF cultures were prepared according to a method reported by Mahida *et al.* (17). The cellular characteristics and culture conditions have been described in our previous report (18).

**Real-time-polymerase chain reaction (PCR).** Expression of human IL-32 $\alpha$  mRNA in samples was assessed by real-time PCR analyses. Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan) with the following primers specific for human IL-32 $\alpha$ ; 5'AGCTGGAGGACGACTTCAAA (nucleotides 192-212, GenBank accession No. BC018782) (19) and 3'AGGTG GTGTCAGTATCTTCA (642-623). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. PCR was conducted using a SYBR-Green PCR Master mix (Applied Biosystems, Foster City, CA). Data were normalized versus  $\beta$ -actin for human IL-32.

**Western blot analyses.** For analysis of IL-32 $\alpha$  protein expression, cells were exposed to cytokines for predetermined periods of time. Cells were then washed with PBS and lysed in SDS sample buffer containing 100  $\mu$ M orthovanadate. For Western blotting, 10  $\mu$ g of protein from each sample was subjected to SDS-PAGE on a 4-20% gradient gel under reducing conditions (20). Biotinylated anti-human IL-32 $\alpha$  antibodies were purchased from R&D Systems and peroxidase-conjugated streptavidine was purchased from Dako Japan (Kyoto, Japan). Subsequently, detection was performed using the enhanced chemiluminescence Western blotting system (Amersham).

For Akt phosphorylation analyses, cells were exposed to cytokines for predetermined periods of time. Antibodies directed against phosphorylated and total Akt were purchased from Cell Signaling Technology (Beverly, MA), and peroxidase-conjugated secondary antibodies were purchased from Amersham (Arlington Heights, IL).

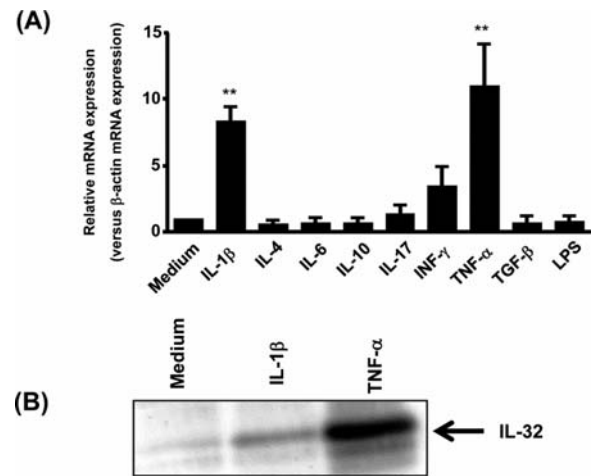


Figure 1. IL-32 $\alpha$  mRNA and protein expression in human colonic SEMFs. (A) IL-32 $\alpha$  mRNA expression. Cells were stimulated with cytokines [IL-1 $\beta$  (10 ng/ml) or other cytokines (100 ng/ml)] for 12 h. IL-32 $\alpha$  mRNA expression was analyzed by real-time PCR analyses. Data are expressed as IL-32 $\alpha$  mRNA relative to  $\beta$ -actin mRNA expression (mean  $\pm$  SD of 4 different experiments); \*\* $P$ <0.01. (B) Intracellular IL-32 $\alpha$  protein expression. Cells were stimulated with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml) for 48 h, and then lysed with lysis buffer. IL-32 $\alpha$  protein was analyzed by Western blotting.

**Adenovirus-mediated gene transfers.** We used a recombinant adenovirus expressing a stable mutant form of I $\kappa$ B $\alpha$  (Ad-I $\kappa$ B $\Delta$ N) (21), a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) (22) and a recombinant adenovirus containing bacterial  $\beta$ -galactosidase cDNA (Ad-LacZ). The stable mutant form of I $\kappa$ B $\alpha$  (I $\kappa$ B $\Delta$ N) lacks 54 NH $_2$ -terminal amino acids of wild-type I $\kappa$ B $\alpha$ , and is neither phosphorylated nor proteolyzed in response to signal induction, but fully inhibits NF- $\kappa$ B activation. The dominant negative mutant c-Jun (TAM67) lacks the transactivational domain of amino acids 3-122 of wild type c-Jun, but it retains the DNA-binding domain. In preliminary experiments, Ad-LacZ infections of colonic myofibroblasts with a multiplicity of infection (MOI) of 10 showed a maximal expression (85% positive) of  $\beta$ -galactosidase. The recombinant adenovirus was transferred into the cells, and cells were made quiescent for 48 h before being assessed for the effects of the transferred gene.

**Statistical analysis.** Statistical significances of differences were determined by the Mann-Whitney U test (StatView Version 4.5). Differences resulting in  $P$ -values <0.05 were considered to be statistically significant.

## Results

To investigate the regulatory mechanisms underlying IL-32 $\alpha$  induction in colonic SEMFs, cells were stimulated with various cytokines for 12 h and IL-32 $\alpha$  mRNA expression was assessed by real-time PCR analyses (Fig. 1A). In these cells, IL-32 $\alpha$  mRNA was weakly expressed without any stimulus, and IL-1 $\beta$  and TNF- $\alpha$  markedly enhanced IL-32 $\alpha$  mRNA expression. IFN- $\gamma$  weakly induced IL-32 $\alpha$  mRNA expression.

Similar results were observed at the protein level. Cells were stimulated for 24 h with IL-1 $\beta$  and TNF- $\alpha$ , and IL-32 $\alpha$

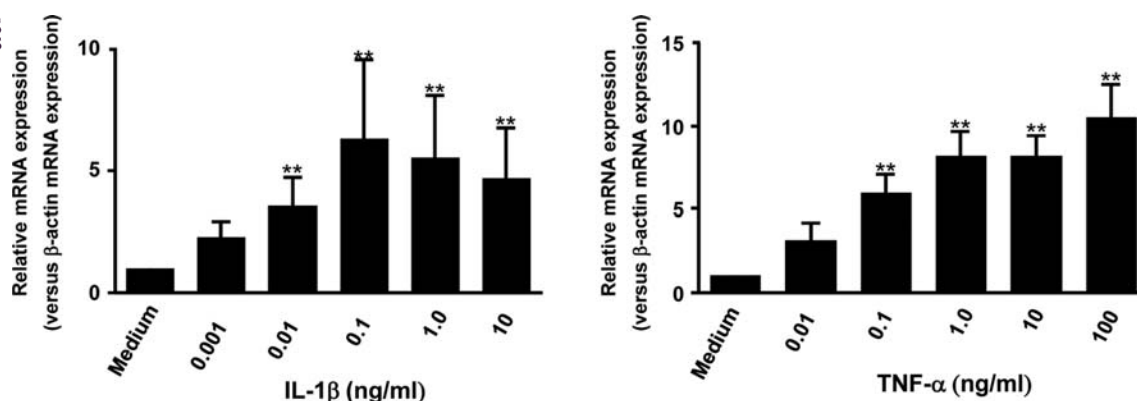


Figure 2. Dose-dependent induction of IL-32 $\alpha$  mRNA in colonic SEMFs. Cells were incubated with different doses of each cytokine, and IL-32 $\alpha$  mRNA expression was determined by real-time PCR analyses. Data are expressed as IL-32 $\alpha$  mRNA expression relative to  $\beta$ -actin mRNA expression (mean  $\pm$  SD of 4 different experiments); \*\*P<0.01.

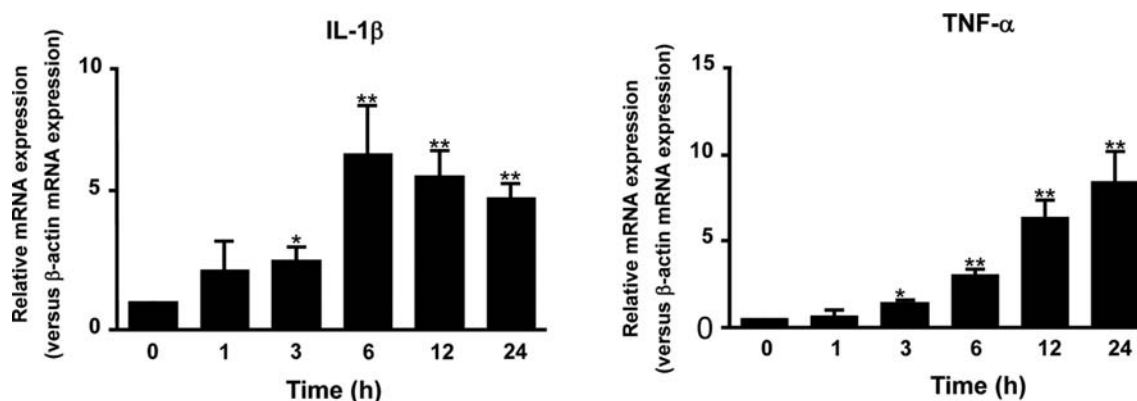


Figure 3. Kinetics of IL-32 $\alpha$  mRNA expression in human pancreatic myofibroblasts. Cells were stimulated with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml) for predetermined times, and IL-32 $\alpha$  mRNA expression was sequentially analyzed by real-time PCR analyses. Data are expressed as IL-32 $\alpha$  mRNA expression relative to  $\beta$ -actin mRNA expression (mean  $\pm$  SD of 4 different experiments); \*\*P<0.01.

protein expression was analyzed by Western blots. IL-32 $\alpha$  was detected as a molecular weight protein of 25 kDa, which is comparable with a previous report (5). Stimulation with IL-1 $\beta$  and TNF- $\alpha$  enhanced intracellular accumulation of IL-32 $\alpha$  protein (Fig. 1B). However, we could not detect the secreted forms of IL-32 $\alpha$  in supernatants.

The effects of IL-1 $\beta$  and TNF- $\alpha$  on IL-32 $\alpha$  mRNA expression were more closely examined. Colonic SEMFs were incubated for 12 h with increasing concentrations of IL-1 $\beta$  and TNF- $\alpha$ , and the IL-32 $\alpha$  mRNA expression was analyzed by real-time PCR. As shown in Fig. 2, these cytokines dose-dependently up-regulated IL-32 $\alpha$  mRNA expression. The IL-1 $\beta$  effect was detected at a concentration as low as 0.01 ng/ml, and reached a maximum at 1.0 ng/ml. The TNF- $\alpha$  effect was observed at a concentration as low as 0.1 ng/ml, and reached a maximum at 100 ng/ml.

The kinetics of IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression were evaluated (Fig. 3). Cells were stimulated with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml) and sequential changes in IL-32 $\alpha$  mRNA expression were determined. IL-1 $\beta$  induced an increase in the accumulation of IL-32 $\alpha$  mRNA, and this reached a maximum 6 h after stimulation. Thereafter, the induced-IL-32 $\alpha$  mRNA levels decreased. TNF- $\alpha$  also

induced a gradual but continuous increase in the accumulation of IL-32 $\alpha$  mRNA for 24 h.

The MAP-kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are implicated in cytokine signaling in various cell types. To investigate the molecular mechanisms underlying IL-32 $\alpha$  induction in colonic SEMFs, we evaluated the effects of the following inhibitors: p42/44 MAP kinase inhibitors (PD98059 and U0126) (23,24), a p38 MAPK inhibitor (SB203580) (25) and a PI3K inhibitor (LY294002) (26). Real-time PCR demonstrated that treatment with the MEK inhibitors (PD98059 and U0126) or a p38 MAPK inhibitor (SB203580) had no effect on IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA (Fig. 4). Contrary to these findings, a PI3K inhibitor, LY294002 (27) significantly blocked the effect of IL-1 $\beta$  and TNF- $\alpha$  on IL-32 $\alpha$  mRNA expression (Fig. 4). These results suggest that PI3K activation is involved in IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression in these cells.

In colonic myofibroblasts, the induction of Akt phosphorylation by IL-1 $\beta$  and TNF- $\alpha$  was evaluated by Western blotting. As shown in Fig. 5, IL-1 $\beta$  and TNF- $\alpha$  induced the Akt phosphorylation as early as 5 min after the stimulation. These data indicate that Akt, a protein kinase recruited by

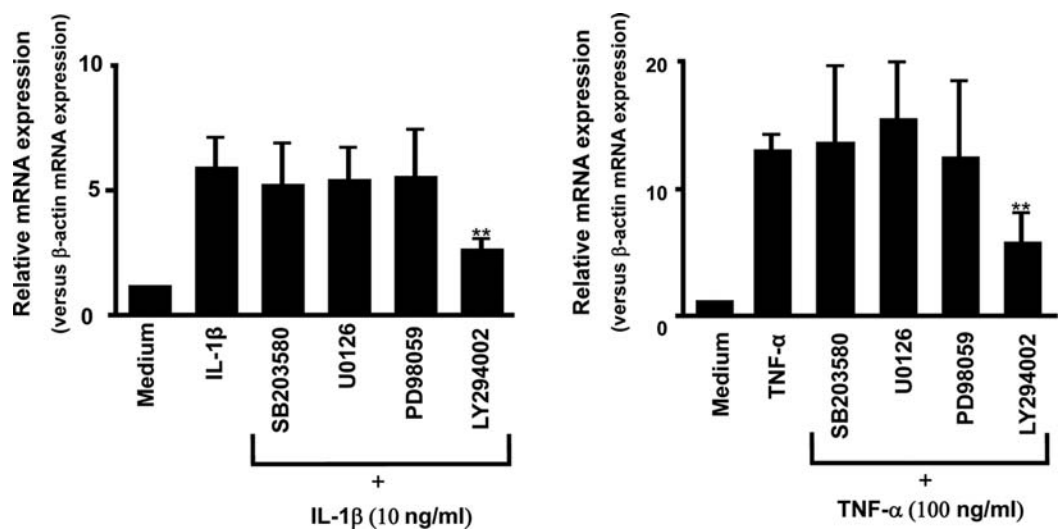


Figure 4. Effects of MAPK inhibitors and a PI3K inhibitor on IL-32 $\alpha$  mRNA expression. Cells were stimulated with each cytokine [IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml)] in the presence or absence of MEK inhibitors [PD98059 (20  $\mu$ M) and U0126 (12.5  $\mu$ M)], a p38 inhibitor [SB203580 (25  $\mu$ M)], and a PI3K inhibitor [LY294002 (25  $\mu$ M)] for 12 h, and then IL-32 $\alpha$  mRNA expression was determined by real-time PCR. Data are expressed as IL-32 $\alpha$  mRNA expression relative to  $\beta$ -actin mRNA expression (mean  $\pm$  SD of 4 different experiments); \*\*P<0.01.

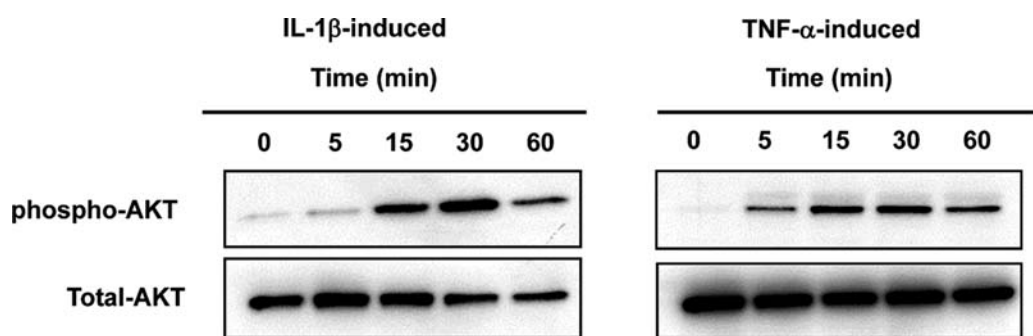


Figure 5. Kinetics of Akt activation in human colonic SEMFs. Cells were stimulated with cytokines [IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml)], and phosphorylated (phospho-) and total Akt were sequentially detected by Western blotting.

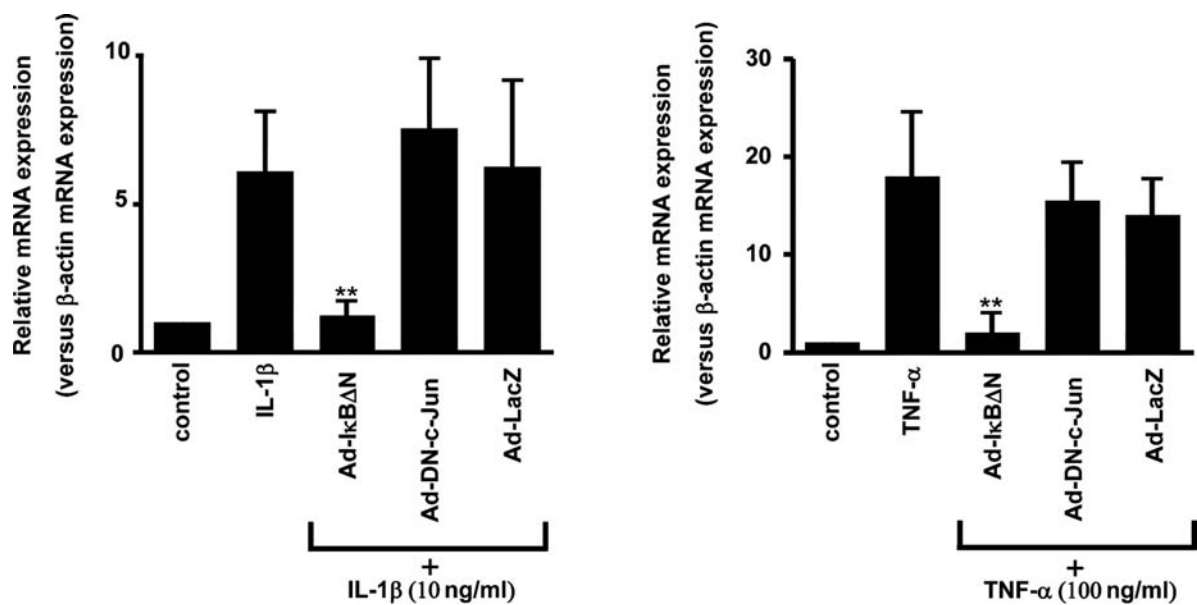


Figure 6. Effects of NF- $\kappa$ B and/or AP-1 inhibition on IL-32 $\alpha$  mRNA expression. Cells were infected with an adenovirus expressing the I $\kappa$ B $\Delta$ N or DN-c-Jun, and after 48 h of infection the cells were stimulated with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml) for 12 h. IL-32 $\alpha$  mRNA expression was determined by real-time PCR analyses. Data are expressed as IL-32 $\alpha$  mRNA expression relative to  $\beta$ -actin mRNA expression (mean  $\pm$  SD of 4 different experiments); \*\*P<0.01. Adenovirus expressing LacZ was used as a negative control.





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To assess the role of the transcription factors NF- $\kappa$ B and AP-1, we evaluated the effects of a recombinant adenovirus containing a stable mutant form of I $\kappa$ B $\alpha$  (Ad-I $\kappa$ B $\Delta$ N) and a dominant negative mutant of c-Jun (Ad-DN-c-Jun) on cytokine-induced IL-32 $\alpha$  mRNA expression. As shown in Fig. 6, cells were infected with a recombinant adenovirus, cultured for 48 h and stimulated for 12 h with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (100 ng/ml), and the expression of IL-32 $\alpha$  mRNAs was determined by real-time PCR. Ad-I $\kappa$ B $\Delta$ N inhibited the effects of both IL-1 $\beta$  and TNF- $\alpha$  on IL-32 $\alpha$  mRNA expression, but Ad-DN-c-Jun did not suppress the effects of IL-1 $\beta$  or TNF- $\alpha$ . Inhibitory effects were not induced by the Ad-LacZ gene, which was used as a negative control. These results suggest that NF- $\kappa$ B plays a role in IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression.

## Discussion

A previous study showed that intestinal epithelial cells are a local site for IL-32 $\alpha$  expression (5,28), and that IL-32 $\alpha$  expression is enhanced in the inflamed mucosa of patients with IBD (28). In this study, we evaluated the IL-32 $\alpha$  expression in colonic SEMFs. This suggests that colonic SEMFs also contribute to the elevation of IL-32 $\alpha$  expression in the IBD mucosa. Since IL-32 acts as a proinflammatory cytokine, which is characterized by induction of the release of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and chemokines) through the NF- $\kappa$ B- and p38 MAPK-activation pathways (4,6), IL-32 $\alpha$  derived from colonic SEMFs stimulates infiltrating immune cells to secrete proinflammatory cytokines and contributes to the deterioration of mucosal inflammation.

The molecular mechanisms underlying IL-32 $\alpha$  induction remain unclear. Previous studies have demonstrated that proinflammatory cytokines such as IL-1 $\beta$ , IL-12, IL-18 and IFN- $\gamma$  are stimulators for IL-32 expression (4,28). In the present study, we demonstrated that IL-1 $\beta$  and TNF- $\alpha$  are potent inducers of IL-32 mRNA expression in human colonic SEMFs. IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression was suppressed by a PI3K-inhibitor (LY294002), but not by p42/44 MAPK-inhibitors (PD98059 and U0126) or a p38 MAPK-inhibitor (SB203580). Furthermore, in these cells IL-1 $\beta$  and TNF- $\alpha$  induced the phosphorylation of Akt, a protein kinase immediately recruited by PI3K activation (29). These observations indicate that the PI3K/Akt pathway contributes to IL-1 $\beta$ - and TNF- $\alpha$ -mediated IL-32 $\alpha$  mRNA induction in colonic SEMFs.

Many cytokine-inducible responses are mediated by DNA binding proteins, such as NF- $\kappa$ B and AP-1. The promoter region of the human IL-32 $\alpha$  gene has consensus binding sites for NF- $\kappa$ B (at bp -638 to -649), suggesting an involvement of NF- $\kappa$ B activation in IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression. To confirm this possibility, we used a recombinant adenovirus expressing a stable mutant form of I $\kappa$ B $\alpha$  (Ad-I $\kappa$ B $\Delta$ N) (21). As shown in Fig. 6, pretreatment with Ad-I $\kappa$ B $\Delta$ N blocked IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression. These data indicate that NF- $\kappa$ B activation plays a role in IL-32 $\alpha$  mRNA induction in colonic SEMFs. In

addition, recent studies indicate that the PI3K/Akt pathway regulates activation of transcription factors, such as NF- $\kappa$ B (27,30-33), suggesting cross-talk between the PI3K/Akt pathway and NF- $\kappa$ B activation in cytokine-induced IL-32 mRNA expression.

Whether IL-32 exerts its biological effects as a secretory cytokine remains unclear, since the IL-32 protein does not possess a typical hydrophobic signal peptide in its N-terminus which is a typical feature of secreted cytokines (7). In Cos7 cells transfected with IL-32 $\alpha$  cDNA, intracellular IL-32 $\alpha$  levels were approximately 7-fold higher compared to secreted IL-32 $\alpha$  levels (4). In contrast, in Cos7 cells transfected with IL-32 $\alpha$  cDNA, the abundance of IL-32 $\alpha$  was comparable in supernatants and lysates (4). In this study, IL-32 $\alpha$  was detected in cell lysate, but was not detected in supernatants. Although it is unclear which of the IL-32 isoform is effectively secreted from particular cell types, it may be that IL-32 $\alpha$  plays a role as a cytoplasmic protein. Recently, Goda *et al* demonstrated that overexpression of intracellular IL-32 $\alpha$  induced apoptosis in HeLa cells (34). These data suggest a role for cytoplasmic IL-32 in cell turnover. Damaged cells are deleted and tissue architecture is restored through apoptosis, and IL-32 $\alpha$  may induce apoptosis in damaged cells at inflammatory sites such as IBD.

In conclusion, we demonstrated that IL-32 $\alpha$  is expressed in human colonic SEMFs. IL-32 $\alpha$  was induced by IL-1 $\beta$  and TNF- $\alpha$ , and was mediated by interactions between the PI3K/Akt-pathway and the NF- $\kappa$ B system. Interestingly, IL-32 $\alpha$  was not secreted by human colonic SEMFs. The role of the cytoplasmic accumulation of IL-32 $\alpha$  in colonic SEMFs should be further investigated.

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