Pet Induces IL1, TNFα, MIF and IL1Ra Through the IKKαβ/NFκB Pathway

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Abstract: Plasmid encoded protein (Pet), a product of *E. coli*, infiltrates leukocyte in experimental models and acts on macrophages as a chemokine. As an immune response orchestrator, macrophages play an important role in inducing pro-inflammatory cytokines. In this work, we tested whether in macrophages Pet stimulates the expression of IL1, TNFα, MIF and the antagonist IL1Ra. We also sought the IKKαβ/NFκB pathway as a possible mediator. We found that indeed Pet increases the mRNA of inflammatory cytokines and the antagonist IL1Ra with different rates and kinetics through the IKKαβ/NFκB pathway, which could be consistent as a factor in chronic inflammation.

Keywords: Plasmid encoded protein, inflammation, signal transduction, IBD, macrophage.

INTRODUCTION

Serin proteases, of the *Enterobacteriaceae* family (SPATES), share homology and are widely distributed. They are secreted by all filogenetic groups, A,B1,B2, D and E; of *E. coli* [1, 2]. Plasmid encoded protein (Pet) is a member of SPATES within the IgA1 proteases subfamily. It is reportedly a byproduct of the O42 strain of enteroaggregative *Escherichia coli,* encoded in a 65MDa plasmid related to protein adherence that has a molecular weight of 108 kDa [3]. Pet acts as a chemokine for macrophages [4] process with great relevance for inflammation. Aerobic cultures of colon obtained from IBD, Crohn's disease and colon cancer patients showed a striking increase in bacteria where half were *E. coli* [5].

By secreting cytokines that stimulate the immune response, macrophages actively participate in inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease [6]. Cytokines, interleukin (IL) IL1, tumor necrosis factor α (TNFα), macrophage migration inhibitory factor (MIF) and the antagonist of interleukin-1 receptor (IL1Ra) have been found to participate in IBD [7, 8], where toll-like receptor (TLR) seem to trigger their secretion. TLR signaling implies the activation of inflammasomes and/or transcription factors such as NFκB to stimulate the production of the inflammatory cytokines and cytokine antagonist mentioned earlier [9-11]. The IKKαβγ are essential components upstream of the NFκB canonical pathway. When the kinase complex is activated, it phosphorylates the IκB, regulatory proteins of NFκB, enabling its release and further translocation to the nucleus**.** Once in the nucleus, NFκB promotes the gene expression of inflammation cytokines or other genes involved in survival, apoptosis and cell division [12].

In the present work we report that Pet induces the expression of cytokines, IL1, TNFα, MIF and the antagonist IL1Ra through the IKKαβ/NFκB pathway. This would suggest that *E. coli* toxins, such as Pet, are important contributing factors to the establishment of inflammation and IBD.

MATERIAL AND METHODS

Cell Cultures

Cells of the murine macrophage line J774 were grown in RPMI medium (cat. R6504 Sigma, USA), supplemented with 2 mM glutamine (Cat. 25030-149, Gibco USA), 1mM non-essential aminoacids (Cat. 111450, Gibco USA), 1mM sodium pyruvate (Cat. 11360070, Gibco USA), and 10% FBS (Cat. 10437010, Gibco USA).

Pet Purification

The Pet clone pCEFN1 was grown in LB broth. The Pet purification procedure was performed as described by Villaseca *et al.* [13] with only slight modifications. The modification consisted of concentrating the protein, using the 50kDa Amicon Ultra-15 membrane (cat. UFC 905024, Millipore USA), before filtration through the anionic exchange column. Pet and media used thorough the experiments contained <0.125EU/ml endotoxin/LPS (data not shown).

Chemotaxis

Chemotaxis experiments were carried out in a Boyden's chamber as described by Morales *et al.* [14]. We used

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macrophages instead of monocytes. However the same conditions in terms of cell number ($8x10^5$), Pet concentration (5.8 µg/ml) and time (90 min), were applied.

RNA Protection Assay (RPA)

The mRNA was obtained from 8 $x10^5$ cells previously incubated with 20µg/ml cycloheximide and then exposed to serum-free media containing 200ng/ml of Pet for 10 and 30 min, and for 1, 3, 6, 12, and 24 h. The cells were then lysed with Trizol (cat. 10296-028 Invitrogen USA**),** and RNA was extracted with RNAeasy mini kit (cat. 74104, Qiagen USA), according to manufacturer instructions.

Complementary RNA for the mCK-2b and mCK-3b Multi-Probe Templates Sets (cat 556156, 556158) were synthesized, starting with 10 ng of probe template, by means of kit 556850, in the presence of ^{33}P-UTP, according to manufacturer instructions. The mix where the reaction took place contained 10 µg of RNA and all procedures followed the manufacturer's instructions on kit 556134 from Pharmingen (BD, USA). Protected fragments were subjected to a 6% PAGE containing 40% urea all dissolved in TBE. The gels were dried, exposed to phosphorimager plates and read by Typhoon equipment for analysis. The quantification was performed by Biorad software Lab Works (Cambridge UK). The results are expressed as a ratio of cDNA of cytokine/cytokine antagonist to cDNA of the housekeeping ribosomal protein L32.

IKKαβ Detection

The J774 cells ($8x10^5$ /dish) were incubated for 36 hours, then exposed at indicated times to serum-free medium containing 200 ng/ml Pet. Cells were lysed with a Laemmli buffer. Proteins were separated by SDS-PAGE at 8% and transferred to PVDF membranes. The IKKαβ were detected by means of the antibody anti- pS176/pS180 IKKαβ (cat. 2697, Cell Signaling USA) and ECL. The control load was monitored with an anti-actin Ab (cat. sc-8432, Sta Cruz Biotechnol USA). NFκB pathway inhibition experiments were performed using 10µM surfactin from *Bacillus subtilis* (cat. S3523, Sigma USA), dissolved in serum-free medium and pre-incubated for 2 h prior to Pet cell exposure.

RESULTS

Under *In vitro* conditions we found that in our cell line model from murine mice Pet indeed is a potent chemoattractant (Fig. **1**), as has been shown for human macrophages.

Pet Induces Expression of MIF, IL1, TNFα, and IL1Ra mRNAs

Pet has been shown to induce leukocyte infiltration in a rat intestinal loop model, suggesting a possible inflammatory process [15]. The inflammation is mainly orchestrated by macrophages and dendritic cells [16], and IL secretion by macrophages one of the major contributing events [17]. Hence, we decided to explore whether Pet was able to induce the production of pro-inflammatory and anti-inflammatory IL in macrophages. In Fig. (**2**) it can be observed that IL1, MIF, TNFα and IL1Ra mRNAs are produced after the addition of Pet to the cells. The IL10 expression was without modification. Basal expression levels are quite different, with TNFα and MIF more abundant than IL1 or IL1Ra. It can also be observed that indeed Pet triggers the three cytokines and the cytokine antagonist with different kinetics. The fastest cytokine induced by Pet was TNFα, starting at 10 min (1.8 fold over basal) and strongly sustained for 3 h, before returning to its basal level. Pet induced the expression of MIF to peak between 20 and 30 min (48% over basal) reaching 60% over basal at 3 h, a response that was sustained for 12h. The kinetics for IL1 expression increases two fold over basal at 30 min, and reaches a four-fold increase by the third hour of stimulation, and finally a slight decrease at 12 h post-stimulation. Pet also induces IL1Ra expression starting at 3 h post-stimulation, and up to a six fold over basal stimulation; by the 12 h post Pet stimulus.

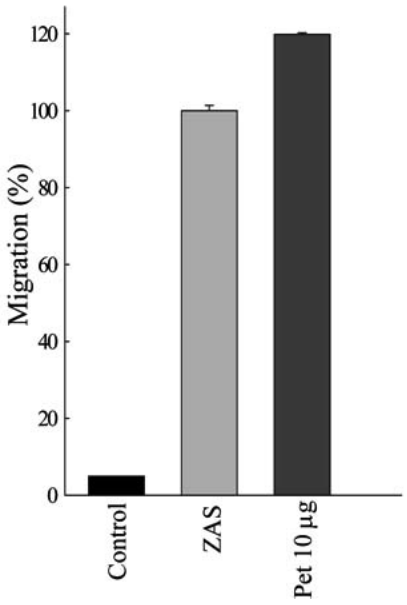

Fig. (1). Pet stimulates chemotaxis in J744 macrophages. Cells were stimulated without or with 10µg of Pet or 40mg /ml of zymosan-serum (ZAS) in serum-free medium. The results are expressed as percentage of Zas-induced response and represent the average ± SE of two different experiments performed by duplicate.

Pet Stimulates the Activation of IKKαβ

NFκB is one of the most common pathways to stimulate the cytokine secretion [18]. The canonical pathways involve the upstream activation of IKKαβ complex by the phosphorylation of the Ser 177 and 181 for IKKβ, and Ser 176 and 180 for IKKα [19]. In order to test whether Pet is capable of stimulating this pathway we exposed macrophages at different times with the toxin and determined the phosphorylation of the kinases with specific antibodies**.** It was observed (Fig. **3**) that IKKαβ phosphorylation was present 5 min after incubation with Pet. The activation peak (four fold) was reached at 1 hour. The activity is brought to almost basal levels after 3 h of incubation with Pet.

NFκB Pathway Mediates Pet-Induced Cytokine and Cytokine Antagonist Expression

To correlate the Pet-induced cytokine and IL1Ra expression with the IKKαβ/NFκB pathway, we used the *Bacillus subtilis* protein surfactin as an inhibitor [20] of the IKKαβ/NFκB pathway, we examined the effects it might

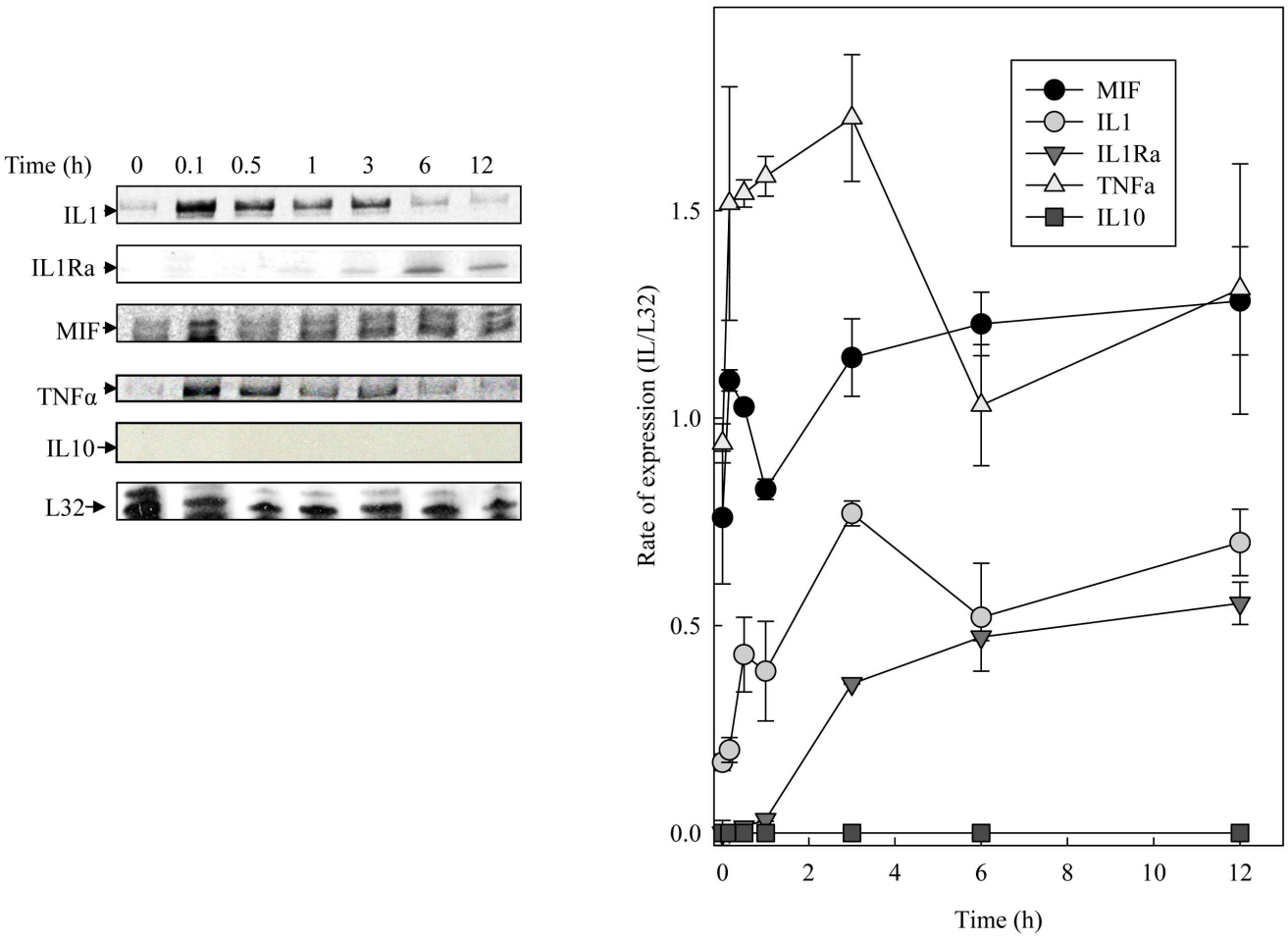

Fig. (2). Pet stimulates MIF, IL1, TNFα and IL1Ra expression in J744 macrophages. Cells were incubated without or with 200 ng Pet prot /ml at indicated times in serum-free medium. Cytokines and antagonist IL1Ra mRNA expression was determined by RPA. The values obtained from densitometries of bands are expressed as the ratio of the gen in study/L32. The results represent the average ± SE of three different experiments.

have on the inflammatory cytokines expression. In Fig. (**4A**) we observed that macrophage pre-incubation with surfactin inhibits the IKKαβ phosphorylation induced by Pet. At the same time, it inhibits the Pet-induced expression of MIF (Fig. **4B**), TNFα (Fig. **4C**), IL1 (Fig. **4D**), and IL1Ra (Fig. **4E**). These data strongly suggest that Pet induced expression of the pro-inflammatory cytokines, MIF, TNFα and IL1, as well as the antagonist IL1Ra is mediated by the IKKαβ/NFκB pathway.

DISCUSSION

Pet Stimulates the Production of Cytokine and IL1Ra mRNAs

Leukocyte infiltration has been observed in a rat intestinal loop model inoculated with Pet, which suggests inflammation of the bowel [15]. *In vitro*, Pet has shown chemotactic properties in human macrophages [4] and in mice macrophages (this work) which could account for the attraction of cells to the site of the tissue damage. Macrophages are key to the immune response [16], hence our interest in knowing how Pet affects their activities. Inflammatory (pro- and anti-inflammatory) cytokine production, such as IL1, TNFα and MIF, IL10, and the antagonist IL1Ra is one of the outstanding events that take place during the macrophage inflammatory response [21, 22]. We found that Pet represented a high intensity inflammatory stimulus with effects ranging from nearly half to six times over basal, for IL1, TNFα, MIF. It also stimulated the production of the antagonist IL1Ra, yet the kinetics of such event would suggest that the extended effect of Pet on the macrophages is due to the autacoid effect, that results from its own IL1 production. The production of IL1Ra regulates local effects of IL1 in endometrial cells [23]. IL1Ra is produced after IL1 induction by a different inflammatory stimulus, such as traumatic brain injury [24]. It is worth noting that Pet did not affect the expression of the anti-inflammatory IL10, which suggests that by generating a cellular response, rather than a Th1 [25], it is behaving as an inflammatory stimulus.

The contribution of each cytokine varies. Perhaps an increase in MIF expression could lead to a chronic stimulation pattern on the macrophage. Mitchell *et al.* [26] have demonstrated that MIF delays macrophage apoptosis; extending its active half-life. As a consequence of half-life

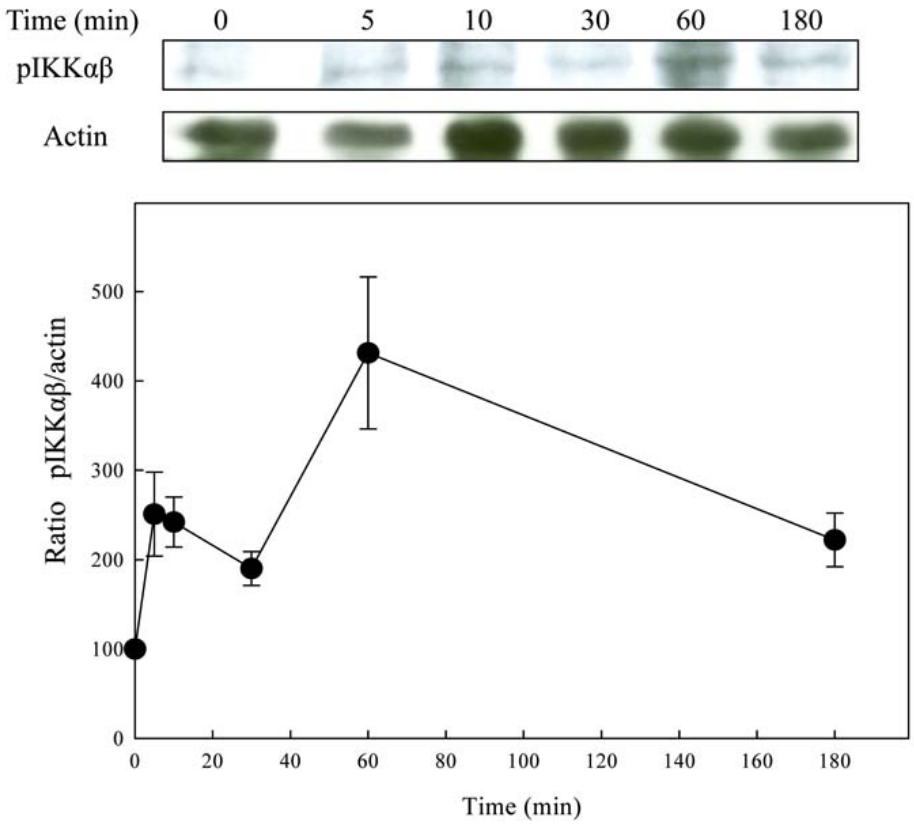

Fig. (3). Pet activates IKKαβ in J744 macrophages. Macrophages were incubated with or without 200 ng Pet prot/ml at the indicated times in serum free medium. Then cells were lysed and subjected to SDS-PAGE electrophoresis, and transferred to PVDF membranes. The resulting pThr-IKKα/pThr-IKKβ was determined as described in Methods. Protein content load was monitored with an anti-actin Ab. The results are representative of 3 different experiments and are expressed as the ratio of pIKKαβ/actin.

increase, other functions are sustained (such as production of IL1), extending the inflammatory process. MIF secretion in macrophages was also induced by the staphylococcal toxic shock syndrome (TSS) toxin 1 (TSST-1) and the streptococcal pyrogenic exotoxin [27]. Interestingly, Staphylococcal and Streptococcal bacteria or their toxins have also been implicated in chronic inflammation pathologies [28].

Pet stimulates an interesting concerting effect on the classical pro-inflammatory cytokines TNFα and IL1. The expression of TNFα started very early and was sustained at least until 3h. While IL1 peaked at 3 hours of Pet stimulation and was sustained for twelve hours. A different kinetics with relevance to the temporary effects was observed in the dextran sulfate sodium colitis-induced mouse model, where TNFα and IL1 were stimulated at acute and chronic models, however, TNFα sustained production was indispensable for the chronic phase [29]. The pattern of IL1/IL1Ra expression was similar to the one triggered by LPS in another report [30]. The balance of IL1/IL1Ra assumes the role of inflammation regulator [31]. In chronic diseases it is important to keep inflammation within a manageable range, hence the need for the IL1/IL1Ra balance; given that IL1Ra binds directly to the IL1 receptor and is capable of avoiding the exacerbation of the response [32, 33]. IBD patients have shown IL1/IL1Ra imbalance [34].

NFκB Mediates the Cytokine and the Antagonist IL1Ra Expression Induced by Pet

NFκB is the classic pathways to stimulate cytokine production. The canonical pathway that leads to NFκB activation involves the participation of IKKαβ [35]. We found that Pet activated IKKαβ and that its activation was sensitive to surfactin. This inhibitor of the NFκB pathway diminished the Pet stimulated cytokine induction, which suggests that the Pet effect is mediated through NFκB. Surfactin abating effects on IL1 induction by LPS have been reported [20]. The effect of surfactin on IL1Ra expression could be a consequence of its effect on IL1 production, i.e. blocking the autacoid effect. The pattern of MIF, TNFα, IL1 induction by Pet, mediated by the NFkB, suggests that Pet could contribute to chronic inflammation. The role of key nodes for IL1, TNFα and NFκB has been found in different chronic inflammatory diseases linked to cancer [36, 37]. Furthermore, SPATES have been found in patients suffering from Crohn's disease and ulcerative colitis [38, 39]. In Crohn's disease and other IBD, the biota population dynamics is disrupted and the homeostasis is broken [39-41]; where *E. coli* is one of the most abundant species [42, 43]. Perhaps its participation is largely due to their secreted toxins.

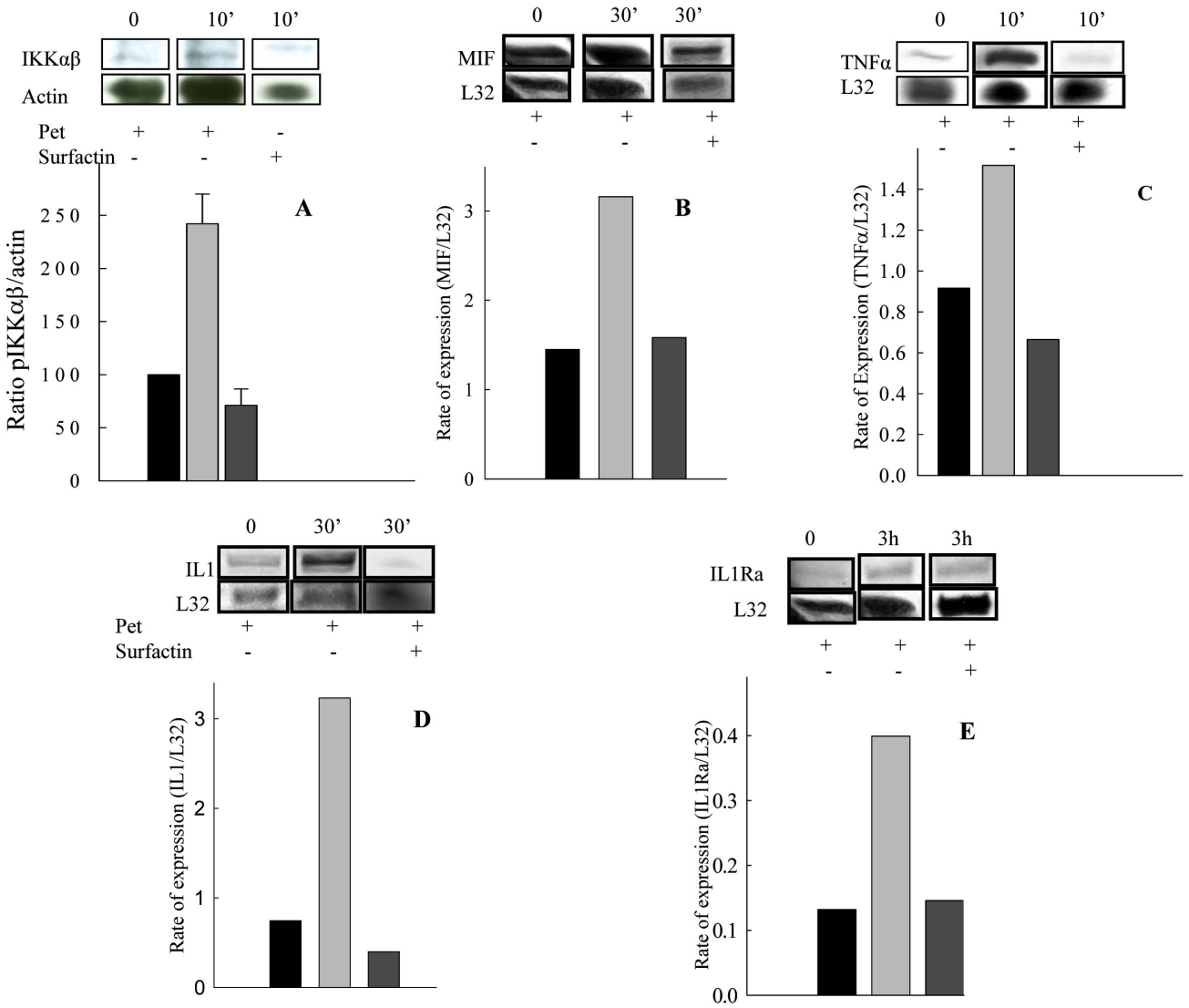

Fig. (4). Surfactin inhibits both IKKαβ activation, and expression of MIF, TNFα, IL1 and IL1Ra. Cells in serum free medium, were pre-incubated with or without 10 mM surfactin for 2 hours and then challenged with 200 ng Pet prot/ml at the indicated times. The results are representative of 2 or 3 different experiments and are expressed as the ratio of pIKKαβ/actin (**A**), or MIF/L32 (**B**), TNFα/L32 (**C**), IL1/L32 (**D**) or IL1Ra/L32 (**E**) expression.

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REFERENCES

[1] Escobar-Páramo P, Clermont O, Blanc-Potard A-B, Bui H, Le Bouguénec C, Denamur E. A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. Mol Biol Evol 2004; 21(6): 1085-94.

[2] Boisen N, Ruiz-Perez F, Scheutz F, Krogfelt K, Nataro JP. Short report: high prevalence of serine protease autotransporter cytotoxins among strains of enteroaggregative *Escherichia coli*. Am J Trop Med Hyg 2009; 80(2): 294-301.

[3] Eslava C, Navarro-García F, Czeczulin JR, Henderson IR, Cravioto A, Nataro JP. Pet, an autotransporter enterotoxin from entero-aggregative *Escherichia coli*. Infect Immun 1998; 66(7): 3155-63.

[4] Hernández-Chiñas U, Gazarian T, Gazarian K, Mendoza-Hernández G, Xicohtencatl-Cortes J, Carlos E. Peptide sequences identified by phage display are immunodominant functional motifs of Pet and Pic serine proteases secreted by *Escherichia coli* and *Shigella flexneri*. Peptides 2009; 30(12): 2127-35.

[5] Kotlowski R, Bernstein CN, Sepehri S, Krause DO. High prevalence of *Escherichia coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease. Gut 2007; 56(5): 669-75.

[6] Danese S, Mantovani A. Inflammatory bowel disease and intestinal cancer: a paradigm of the Yin-Yang interplay between inflammation and cancer. Oncogene 2010; 29(23): 3313-23.

[7] Sanchez-Muñoz F. Role of cytokines in inflammatory bowel disease. World J Gastroenterol. 2008; 14(27): 4280-8.

[8] Shah YM, Ito S, Morimura K, *et al*. Hypoxia-inducible factor augments experimental colitis through an MIF-dependent inflammatory signaling cascade. Gastroenterology 2008; 134(7): 2036-48.

[9] Martinon F, Chen X, Lee A-H, Glimcher LH. TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. [Internet]. Nat Immunol 2010; 11(5): 411-8.

[10] Tsuchiya A, Imai K, Asamitsu K, Waguri-nagaya Y, Otsuka T. Inhibition of inflammatory cytokine production from rheumatoid synovial fibroblasts by a Novel I B Kinase Inhibitor. J Pharmacol Exp Ther 2010; 333(1): 236-43.

[11] Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol 2006; 290(4): L622-L45.

[12] Häcker H, Karin M. Regulation and function of IKK and IKK-related kinases. Science's STKE 2006; 2006(357): re13

[13] Villaseca JM, Navarro-García F, Mendoza-Hernández G, Nataro JP, Cravioto A, Eslava C. Pet toxin from enteroaggregative *Escherichia coli* produces cellular damage associated with fodrin disruption. Infect Immun 2000; 68(10): 5920-7.

[14] Morales ME, Rico G, Gómez JL, *et al*. Could the homologous sequence of anti-inflammatory pentapeptide (MLIF) produced by *Entamoeba histolytica* in the N protein of rabies virus affect the inflammatory process? Parasitol Res 2006; 98(3): 232-6

[15] Sainz T, Perez J, Fresan MC, *et al*. Histological Alterations and Immune Response Induced by Pet Toxin During Colonization with Enteroaggregative *Escherichia coli* (EAEC) in a Mouse Model Infection. J Microbiol 2002; 40(2): 91-7.

[16] Bilsborough J, Viney JL. Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease [Internet]. Gastroenterology 2004; 127(1): 300-9.

[17] Kaiser P, Rothwell L, Avery S, Balu S. Evolution of the interleukins. Dev Comp Immunol 2004; 28(5): 375-94.

[18] Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 2009; 1(6): a001651.

[19] Israël A. The IKK Complex, a Central Regulator of NF-kappaB Activation. Cold Spring Harb Perspect Biol 2010; 2(3): a000158.

[20] Byeon SE, Lee YG, Kim BH, *et al*. Surfactin blocks NO production in lipopolysaccharide-activated macrophages by inhibiting NF-kappaB activation. J Microbiol Biotechnol 2008; 18(12): 1984-9.

[21] Roger T, Glauser MP, Calandra T. Macrophage migration inhibitory factor (MIF) modulates innate immune responses induced by endotoxin and Gram-negative bacteria. J Endotox Res 2001; 7(6): 456-60.

[22] Kiemer AK, Vollmar AM. The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. Ann Rheum Dis 2001; 60(Suppl 3): iii68-70.

[23] Bellehumeur C, Blanchet J, Fontaine J-Y, Bourcier N, Akoum A. Interleukin 1 regulates its own receptors in human endometrial cells *via* distinct mechanisms. Hum Reprod (Oxford, England) 2009; 24(9): 2193-204.

[24] Helmy A, Carpenter KLH, Menon DK, Pickard JD, Hutchinson PJ a. The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production. J Cereb Blood Flow Metab 2011; 31(2): 658-70.

[25] Goettel JA, Algood SHM, Olivares-Villagómez D, *et al*. KSR1 protects from interleukin-10 deficiency-induced colitis in mice by suppressing T-lymphocyte interferon-γ production. Gastroenterology 2011; 140(1): 265-74.

[26] Mitchell R, Liao H, Chesney J, *et al*. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci USA 2002; 99(1): 345-50.

[27] Calandra T, Spiegel LA, Metz CN, Bucala R. Macrophage migration inhibitory factor is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria. Proc Natl Acad Sci USA 1998; 95(19): 11383-8.

[28] Tripathi A, Conley DB, Grammer LC, *et al*. Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis. The Laryngoscope 2004; 114(10): 1822-6.

[29] Kojouharoff G, Hans W, Obermeier F, *et al*. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. Clin Exp Immunol 1997; 107(2): 353-8.

[30] Molnarfi N, Gruaz L, Dayer J-M, Burger D. Opposite regulation of IL-1beta and secreted IL-1 receptor antagonist production by phosphatidylinositide-3 kinases in human monocytes activated by lipopolysaccharides or contact with T cells. J Immunol 2007; 178(1): 446-54.

[31] Arend WP. The balance between IL-1 and IL-1Ra in disease. Cytokine Growth Factor Rev 2002; 13(4-5): 323-40.

[32] Papadakis K. Role of cytokines in the pathogenesis of inflammatory bowel disease. Annu Rev Med 2000; 98(1): 1010-298.

[33] van den Berg WB. Arguments for interleukin 1 as a target in chronic arthritis. Ann Rheum Dis 2000; 59(Suppl I): i81-4.

[34] Ludwiczek O, Vannier E, Borggraefe I, *et al*. Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. Clin Exp Immunol 2004; 138(2): 323-9.

[35] Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol 2002; 3(3): 221-7.

[36] Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005; 5(10): 749-59.

[37] Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010; 140(6): 883-99.

[38] Rhodes JM. The role of *Escherichia coli* in inflammatory bowel disease. Gut 2007; 56(5): 610-2.

[39] Eckburg PB, Relman DA. The role of microbes in Crohn's disease. Clin Infect Dis 2007; 44: 256-62.

[40] Eckburg PB, Bik EM, Bernstein CN, *et al*. Diversity of the human intestinal microbial flora. Science 2005; 308(5728): 1635-8.

[41] Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008; 134(2): 577-94.

[42] Darfeuille-Michaud a, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P. Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. Gastroenterology 1998; 115(6): 1405-13.

[43] Schwiertz A, Jacobi M, Frick J-S, Richter M, Rusch K, Köhler H. Microbiota in Pediatric Inflammatory Bowel Disease. J Pediatr 2010; 157(2): 240-4.

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