LPS may enhance expression and release of HMGB1 in human nasal epithelial cells in vitro

Espressione e rilascio della proteina HMGB1 da coltura di cellule epiteliali nasali dopo stimolazione con LPS

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SUMMARY

Chronic rhinosinusitis with nasal polyps is a common disease with still unclear pathophysiologic mechanisms. The airway epithelial barrier has been shown to be involved in different chronic disorders, including rhinitis, nasal polyposis and asthma. High mobility group box 1 (HMGB1), a primarily nuclear protein, is involved in the induction of airway inflammation in patients with chronic rhinosinusitis, allergy, asthma and COPD. Pathogen-derived lipopolysaccharide is widely used as a trigger for inflammation. However, the molecular dialogue between LPS and HMGB1 in the delayed inflammatory processes remains to be explored, and the regulation of HMGB1 release through LPS from epithelial cells has not been extensively studied in patients with chronic rhinosinusitis and nasal polyps. The objective of the present study was to investigate the relocation of HMGB1 in LPS-induced human nasal epithelial cells in vitro. We obtained epithelial cells of nasal polyps from 10 patients requiring surgery for sinusitis at the ENT Department of the Chinese PLA General Hospital. The primary cultured human nasal epithelial (HNE) cells were stimulated with LPS. The expression and translocation of HMGB1 in intracellular and culture supernatants were determined using Western blot and immunofluorescence assay. HMGB1 protein was released in a time-dependent fashion in culture supernatants: in fact, expression of HMGB1 protein in HNE cells showed no significant changes at 0-24 h after exposure to 100 µg/ml LPS, but increased significantly at 48 and 72 hr. Immunofluorescence analysis revealed the transfer of HMGB1 from nuclei to cytoplasm in response to LPS exposure after 24 hr. These data reveal a hitherto unrecognized association between HMGB1 and LPS in human nasal epithelial cells. LPS can affect HMGB1 translocation and release, suggesting the involvement of HMGB1, through inflammatory mediators, in chronic rhinosinusitis with nasal polyps.

KEY WORDS: HMGB1 protein • Lipopolysaccharides • Nasal polyps • Human nasal epithelial cells • Primary cell culture

RIASSUNTO

La rinosinusite cronica e la poliposi nasale sono patologie frequenti con un meccanismo fisiopatologico non del tutto chiarito. La barriera epiteliale delle vie respiratorie sembra essere coinvolta in diverse patologie croniche come la rinite, la poliposi nasale e l’asma. HMGB1, proteina espressa originalmente a livello nucleare, è coinvolta nell’induzione dell’infiammazione delle vie aeree nei pazienti affetti da rinosinusiti croniche, rinite allergica, asma e COPD. I Lipopolisaccaridi batterici sono ampiamente utilizzati come trigger dell’infiammazione. Tuttavia, il dialogo molecolare tra LPS e HMGB1, nella fase tardiva del processo infiammatorio, resta da approfondire. La regolazione del rilascio di HMGB1 dopo stimolazione con LPS dalle celle epiteliali non è ancora stata studiata in modo esaustivo in pazienti con diagnosi di rinosinusite cronica con poliposi nasale. L’obiettivo di questo studio è stato quello di analizzare in vitro la localizzazione di HMGB1 in colture di cellule epiteliali nasali umane dopo stimolazione con LPS. Abbiamo prelevato cellule epiteliali di polipi nasali da 10 pazienti sottoposti a chirurgia per sinusite cronica presso il Dipartimento di O.R.L. del PLA General Hospital di Pechino. Abbiamo stimolato la coltura primaria di cellule epiteliali nasali umane con LPS. L’espressione di HMGB1 e la traslocazione intracellulare e nel surnatante della coltura sono stati determinati usando la tecnica Western Blot e la determinazione in immunofluorescenza. L’espressione della proteina HMGB1 nelle cellule epiteliali nasali umane non ha mostrato variazioni significative a 0-24 h dall’esposizione a 100 µg/ml di LPS, ma è aumentata significativamente a 48 e 72 h. HMGB1 è rilasciata nel surnatante in modo tempo-dipendente. Le analisi di immunofluorescenza hanno mostrato il trasferimento di HMGB1 dal nucleo al citoplasma in risposta all’esposizione ad LPS dopo 24 ore. I nostri risultati hanno evidenziato un’associazione tra HMGB1 e LPS a tutt’oggi non dimostrata. I LPS possono pertanto ritenersi implicati nella traslocazione e nel rilascio di HMGB1 dalle cellule epiteliali nasali umane, suggerendo il coinvolgimento di HMGB1 come mediatore infiammatorio tardivo nelle rinosinusiti croniche associate o meno a poliposi nasale.

PAROLE CHIAVE: Proteina HMGB1 • Lipopolisaccaridi • Polipi nasali • Cellule epiteliali nasali umane • Coltura cellulare primitiva

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Expression and release of HMGB1 in human nasal epithelial cells in vitro

Introduction

Inflammatory cytokines are important factors that mediate inflammation, and have the potential to initiate and maintain nasal and sinus mucosa responses to different kinds of stimuli. Nasal polyps are the consequence of persistent inflammatory and remodeling responses in several chronic inflammatory diseases. Current treatments relieve symptoms, but do not resolve the high incidence of recurrences, which underlines the need for specific research correlating novel molecular targets, inflammation and nasal mucosa function.

Previous studies have shown that high mobility group box 1 (HMGB1) protein, as the class of “alarmins”, participates in the innate and adaptive immune responses such as chronic obstructive pulmonary disease (COPD), asthma, sepsis, cystic fibrosis (CF) and systemic lupus erythematosus. There is evidence that the release of damage-associated molecular patterns (DAMPs) in the epithelium, such as HMGB1, may evoke inflammatory responses in the lower airways and local nasal mucosa. The initial phase of HMGB1 secretion requires an inflammatory signal such as lipopolysaccharides (LPS), IL-1 or TNF-α for monococytes or macrophages. LPS, a component of the outer membrane of Gram-negative bacteria, is suggested to participate in interacting and activating immune cells where it translocates nuclear HMGB1 to the cytoplasm and extracellular space. In turn, these cells respond to HMGB1 by increasing their migration, proliferation and activating downstream cytokines release. Until now, the role of HMGB1 release and related molecular mechanisms have not been elucidated completely.

Recently, our studies have demonstrated that nasal polyps augment HMGB1 expression both in the epithelium and extracellular matrix, which indicates that HMGB1 may play an important role in CRSwNP. However, the mechanism of HMGB1 protein in CRSwNP remains unclear. We designed a study to further understand the relationships between LPS and HMGB1 in human nasal epithelial (HNE) cells. In primary culture, HNE cells are able to differentiate into ciliated cells. In this study, we stimulated epithelial cells with LPS in vitro and observed the expression and translocation of HMGB1 by immunofluorescence assay and Western blot.

Materials and methods

Sample collection and cell culture

We obtained epithelial cells of nasal polyps and paranasal sinus mucosa from 10 patients requiring surgery for their sinusitis, excluding cases with non-invasive fungal sinusitis, chronic obstructive pulmonary disease, cystic fibrosis, primary ciliary dyskinesia (PCD) or severe asthma. This study was approved by the ethics committee of Chinese PLA General Hospital, and all patients gave their informed consent before recruitment in the study.

Fresly removed tissue was placed in Dulbecco’s phosphate buffer (Ca²⁺ and Mg²⁺ free) before transfer to the laboratory. We digested the tissue in DMEM/F12 containing 0.1% protease type XIV (Sigma-Aldrich Corp), 0.1 mg/ml of deoxyribonuclease (Sigma-Aldrich Corp), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 0.25 µg/ml amphotericin B for 18-24 hours. 10% foetal bovine serum (FBS) was added to neutralize the protease. After centrifuging, cells were counted and then plated on collagen type IV (Sigma) coated tissue culture 24- or 48- well plates in 10% FBS+BE GM (Gibco) (Table I) for 2 to 3 days. Next, we cultured the cells in serum-free modified medium (HD MEM+BE BM 1:1). Cells were grown submersed until almost confluent in a humidified chamber with 95% air and 5% CO₂ at 37 °C. The culture medium was changed every day. LPS was added during mucociliary differentiation (continuous treatment). For observing mucociliary differentiation, cells were also directly seeded on 6.5 mm transwell plates and maintained in air–liquid interface culture (ALI).

LPS solution and stimulation

LPS (Escherichia coli O111:B4; Sigma, St. Louis, MO) was dissolved in BEBM medium at stock concentrations of 5 mg/ml, stored at -80 °C. Immediately before each experiment, an aliquot of LPS was thawed and diluted to working concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml. At 85-95% confluence, cells were treated with the three indicated LPS concentrations. The levels of HMGB1 protein in epithelial cells and supernatants were assessed after 0, 12, 24, 48 and 72 hr by Western blot.

Table I. Culture medium composition.

| Culture medium     | BEBM       | H-DMEM+BE BM (1:1) |
|--------------------|------------|--------------------|
| Insulin (µg/ml)    | 5          | 5                  |
| Transferrin (µg/ml)| 10         | 10                 |
| Hydrocortisone (µM)| 1.4        | 1.4                |
| Epidermal growth factor (ng/ml)| 0.5 | 0.01               |
| Epinephrine (µM)  | 2.7        | 2.7                |
| Triiodothyronine (nM)| 9.7    | 9.7                |
| Bovine pituitary extract (%) | 0.26 | 0.26             |
| Retinoic acid (mM) | 0.3        | 50                 |
| Penicillin G+ streptomycin sulphate | 100 U/ml+100 µg/ml | 100 U/ml+100 µg/ml |
| Gentamicin b+amphotericin b | 50 µg/ml+0.25 µg/ml | -                 |
Scanning electron microscopy
The air-liquid interface membranes were dehydrated in a progression of increasing ethanol, dried in CO₂, sputter-coated with gold palladium and then examined in a scanning electron microscope (SEM).

Cell viability assay
Because HMGB1 is not only secreted by activated innate immune cells, but also leaked by necrotic cells, prior to immunofluorescence and Western blot experiments the viability of cells used for experiments was assessed by 4% trypan blue exclusion and the live/dead viability determined. Only population of cells with a viability > 95% were used for experiments.

Immunofluorescence microscopy
Cell cultures were perfused with DPBS to remove the dead cells, and fixed in 4% paraformaldehyde for at least 20 min. Nonspecific staining was blocked with 5% goat serum, 3% bovine serum albumin (BSA) and 0.3 Triton® X-100 at room temperature for 30 min. Next, cells were incubated in 1% BSA-HMGB1 rabbit polyclonal antibody (Abcam, dilution 1:300) or β-tubulin mouse monoclonal antibody (Sigma, dilution 1:200) overnight at 4 °C. After three washes with PBS, the cells were incubated in alexa fluor® 488 goat anti-rabbit IgG and alexa fluor® 594 goat anti-mouse IgG (Invitrogen, dilution 1:1000) at room temperature for 60 min. Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI). Negative controls were processed in parallel without a primary antibody incubation step. Immunofluorescent photographs were taken with Leica fluorescence microscope.

HMGB1 protein Western blot analysis
Total protein extracts were prepared from epithelial cells in RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell culture supernatants were collected after elimination, filtration (0.22 µm filter) and concentration using Amicon Ultra centrifugal filter devices (Millipore). Protein samples were quantified using the BCA assay kit (DingGuo). Briefly, equal amounts of cellular proteins were boiled and then loaded onto a 12% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk in TBST, the membrane was incubated with primary antibodies specific for HMGB1 mouse monoclonal antibody (Abcam, dilution 1:500) and β-actin mouse monoclonal antibody (Santa Cruz, dilution 1:1000) overnight. Subsequently, the membrane was incubated with the appropriate secondary antibody (Jackson ImmunoResearch Laboratories, dilution 1:3000) for 2 hr, and immunoreactive bands were visualized using a chemiluminescence ECL kit (Pierce). The relative band intensity was quantified by using Quantity One analyzer software.

Statistical analysis
All statistical analysis was performed using SPSS13.0 statistical analysis software. Results are presented as the means ± SD of 5 independent experiments. Those without significantly deviation from the normally distributed population were selected for one-way ANOVA analyses to determine significant differences among groups. When the ANOVA was significant, post hoc testing of difference was performed using Tukey’s test. A p value < 0.05 was considered statistically significant.

Results
Morphological characterization of HNE cells in vitro
To characterize the effect of inflammatory mediator LPS on HMGB1 expression in HNE cells, we first established primary cultures of human nasal epithelial (HNE) cells, which has greatly facilitated the study of upper airway diseases. HNE cells remained in a differentiated state (Figs. 1A
left, 1B) in submerged cultivation. Demonstration of β-tubulin by immunofluorescence indicated an intact monolayer with a differentiated cell population. On the other hand, with SEM the epithelial surface showed ciliated cells and microvilli cells for an ALI cultivation (Fig. 1 A right), which was similar to that reported in the literature 17.

**LPS alters HMGB1 protein localization in HNE cells**

Immunofluorescence and fluorescence microscopes were used to confirm the location of intracellular HMGB1 protein in LPS-induced HNE cells. The nuclei stained strongly, while no cytoplasm stained with red at 0 or 12 h, indicating that HMGB1 is distributed mainly in the nuclei (Fig. 2A-B). After stimulation, the cytoplasmic stain increased gradually from 24 hr and enhanced from 48 to 72 hr, and nuclear staining decreased gradually at the same time (Fig. 3C-E). These indicated that LPS induced HMGB1 translocation to the cytoplasm and extracellular release.

**LPS stimulated a time-dependent production of HMGB1 protein in HNE cells**

Although HMGB1 location changed following stimulation with LPS, it is still unknown if the LPS stimulus affects HMGB1 production in HNE cells. To explore this possibility, HNE cells were stimulated with 100 µg/ml LPS. The result was confirmed in Western blot. LPS induced an increase level of HMGB1 in HNE cells in a closely related time-dependent manner (Fig. 3). The level of HMGB1 protein in HNE cells showed no significant change at 0-24 hr, but HMGB1 protein levels at 48 hr were higher than at 0 hr, as well as for HMGB1 level at 72 hr.

**Release of HMGB1 protein from HNE cells**

Secreted HMGB1 in the cultured supernatant of the LPS-treated HNE cells was detectable after 12 hr, and the concentration of HMGB1 significantly increased at 24 hr after onset of treatment and continued to increase steadily (Fig. 4). The HMGB1 concentration in the culture supernatant was significantly higher at 48 hr and 72 hr compared to 0, 12 and 24 hr (p < 0.001 for each time point).

**Discussion**

Infections (viral, bacterial and fungal) under physiological conditions are readily eliminated without involvement of the adaptive immune system at the mucosal lining of the upper airways which is the first-line defense mechanism against pathogens and antigens 19. Airway diseases such as rhinosinusitis and asthma are characterized by impaired airway epithelium immune barrier function 19 20. LPS has emerged as a key regulator of inflammation; its exposure level is implicated in airway epithelium integrity, and in initiation of airway disease and development 21 22. It is
already known that DAMPs, such as HMGB1, may induce the selective recruitment of immune cells and lead to airway inflammation, and as such are important events in the progression of the inflammation response. To better understand the pathophysiological role of HMGB1 in inflammation, we used primary cultures of human nasal epithelial (HNE) cells, and demonstrated LPS-induced active translocation and release of HMGB1 in a time-dependent manner.

In the present study, to ensure that HMGB1 protein was secreted from viable HNE cells, we used trypan blue exclusion to evaluate cell viability after LPS stimulation. When exposed to 10-100 µg/ml LPS, the viability of cells was not affected. This study documented for the first time the presence of HMGB1 immunostaining in the nuclei of HNE cells from nasal polyps; after LPS stimulation, translocation of HMGB1 from nuclei to cytoplasm occurred, suggesting that HNE cells represent a potential source of the secreted form of HMGB1 in the airways. This finding was in agreement with the results of Western blot analysis. In line with our results, the level of intracellular HMGB1 protein did not change in the early stages (0-24 hr) of stimulation with LPS, but increased significantly at 48 hr and maintained a higher level for up to 72 hr. Furthermore, HMGB1 protein was also released in the culture supernatant from viable HNE cells in a time-dependent fashion. The extracellular HMGB1 protein was significantly elevated at 24-72 hr. These findings are consistent with prior reports, indicating that HMGB1 is a late proinflammatory cytokine and differs from the early release of proinflammatory cytokines, such as TNF (1-2 hr), IL-1 (4-6 hr) and IFN-γ (4-6 hr) by LPS. Our results suggest that after the export of cytoplasmic HMGB1 to the culture medium, intra-nuclear HMGB1 is re-synthesized and relocated, keeping its level high.

Alternations in the structure and function of the nasal epithelial cells change the mechanical properties of the airway barrier and might also drive inflammation. Nasal epithelial cells are a major target for LPS, and toll-like receptors (TLR) -2, -4 and -9, members of the larger family of TLRs, have been demonstrated to play a role in nasal epithelial cells and related nasal mucosa diseases. They belong to pattern-recognition receptors (PRPs) and directly recognize PAMP on pathogens. Via TLR-4 signaling, bacterial products (e.g. LPS) may subsequently trigger the intracellular signaling pathway, including the MAPK-p38 and NF-κB pathways. Many inflammatory responses to LPS are mediated by activation of the two pathways and enhance the transcription of HMGB1 products of the activated macrophage. The mechanism of HMGB1 transfer from the nuclei to special cytoplasmic organelles, namely secretory lysosomes, is related to acetylation of specific lysine residues. Following this, HMGB1 accumulates in the cytoplasm with its nuclear reentry blocked. Once released
into the extracellular medium, HMGB1 may exert its effects by binding to its receptors, RAGE, TLR-2, TLR-4 and TLR-9. Since HMGB1-induced activation of NF-κB is known to involve TLR-2 and TLR-4, we hypothesize that HMGB1 may activate the NF-κB signaling pathway to cause cytokine production and thereby indirectly promote dysfunction of the epithelial cell barrier. We have previously observed that cytokines IL-5, IL-8 and TNF-α may interfere with the expression and the localization of HMGB1 in nasal polyps. Clearly, more experimental investigations will be required to increase our understanding of these interacting networks, and especially the regulatory pathways of HMGB1 in HNE cells.

Taken together, the nasal epithelium is more than a physical barrier, and is critical in regulating nasal and sinus mucosa homeostasis. This study demonstrated that HNE cells are a source of HMGB1 and that LPS triggers active release of HMGB1. Our findings suggested that LPS plays a potentially important role in the induction and prolongation of inflammatory processes by inducing HMGB1 release and “recycle” of injurious proinflammatory mediators. Understanding the mechanisms of infection- or injury-elicted inflammatory responses directly or indirectly through HMGB1 may provide important insights into the pathogenesis associated with end products in chronic obstructive pulmonary disease.

References

1. Van CK, Zhang N, Gevaert P. Pathogenesis of chronic rhinosinusitis: inflammation. J Allergy Clin Immunol 2011;128:728-32.

2. Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Rhinol Suppl 2012;50:1-12.

3. Passali D, Berstein JM, Passali FM, et al. Treatment of recurrent chronic hyper-plastic sinusitis with nasal polypsis. Arch Otolaryngol Head Neck Surg 2003;129:656-9.

4. Rowe-Jones JM, Medcalf M, Durham SR, et al. Functional endoscopic sinus surgery: 5 year follow-up and results of a prospective, randomised, stratified, double-blind, placebo controlled study of postoperative fluticasone propionate aqueous nasal spray. Rhinology 2005;43:2-10.

5. Nassima F, Séverine L, Alexander K. Expression of high mobility group box 1 and of receptor for advanced glycation end products in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010;181:917-27.

6. Shim EJ, Chun E, Lee HS, et al. The role of high-mobility group box-1 (HMGB1) in the pathogenesis of asthma. Clin Exp Allergy 2012;42:958-65.

7. Rowe SM, Jackson PL, Liu G, et al. Potential role of high-mobility group box 1 in cystic fibrosis airway disease. Am J Respir Crit Care Med 2008;178:922-31.

8. Wang H, Bloom O, Zhang M, et al. HMGB-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248-51.

9. Li J, Xie H, Wen T, et al. Expression of high mobility group box chromosomal protein 1 and its modulating effects on downstream cytokines in systemic lupus erythematosus. J Rheumatol 2010;37:766-75.

10. Daly KA, Liu S, Agrawal V, et al. Damage associated molecular patterns within xenogeneic biologic scaffolds and their effects on host remodeling. Biomaterials 2012;33:91-101.

11. Liu Y, Wang ZB, Liu Z, et al. The expression of HMGB1 in nasal mucosa of allergic rhinitis patients and its significance. Zhong Hua Xue Yi Xue Za Zhi 2010;26:651-4.

12. Ferhani N, Letuve S, Kozhich A, et al. Expression of high-mobility group box 1 and of receptor for advanced glycation end products in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010;181:917-27.

13. Wu CX, Sun H, Liu Q. LPS Induces HMGB1 relocation and release by activating the NF-κB-CBP signal transduction pathway in the murine macrophage-like cell line RAW264.7. J Surg Res 2012;175:88-100.

14. Passali D, Kern E, Lei Chen R. High mobility group box 1 (HMGB1): a new protein in the pathogenesis of ENT inflammatory and infectious diseases. Acta Otorhinolaryngol Ital 2012;32:46-7.

15. Bellussi LM, Chen L, Chen D, et al. The role of high mobility group box 1 chromosomal protein in the pathogenesis of chronic sinusitis and nasal polypsis. Acta Otorhinolaryngol Ital 2012;32:386-92.

16. Million K, Tourner F, Houcine O, et al. Effects of retinoic acid receptor-selective agonists on human nasal epithelial cell differentiation. Am J Respir Cell Mol Biol 2001;25:744-50.

17. Lai Y, Chen B, Shi J, et al. Inflammation-mediated upregulation of centrosomal protein 110, a negative modulator of ciliogenesis, in patients with chronic rhino-sinusitis. J Allergy Clin Immunol 2011;128:1207-15.

18. Masaki T, Kojima T, Okabayashi T, et al. A nuclear factor-kappaB signaling pathway via protein kinase D delta regulates replication of respiratory syncytial virus in polarized normal human nasal epithelial cells. Mol Biol Cell 2011;22:2144-56.

19. Tieu DD, Kern RC, Schleimer RP. Alterations in epithelial barrier function and host defense responses in chronic rhinosinusitis. J Allergy Clin Immunol 2009;124:37-42.

20. Xiao C, Puddicombe SM, Field S, et al. Defective epithelial barrier function in asthma. J Allergy Clin Immunol 2011;128:549-56, e1-12.

21. Thorley AJ, Ford PA, Giembycz MA, et al. Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages. J Immunol 2007;178:463-73.

22. Eisenbarth SC. Dependent T helper cell type responses to inhaled antigen. J Exp Med 2002;196:1645-51.

23. Wang H, Bloom O, Zhang M, et al. HMGB-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248-51.

24. Wang H, Yang H, Czura CJ, et al. HMGB1 as a late mediator of lethal systemic inflammation. Am J Respir Crit Care Med 2001;164:1768-73.

25. Deriy LV, Beno DW, Ubing MR, et al. Splenectomy ablates endotoxin-induced IFN-γ response in rats. Shock 2002;17:312-5.
26 Lauriello M, Micera A, Muzi P, et al. TLR4 and TLR9 expression in different phenotypes of rhinitis. Int J Otolaryngol 2012;2012:925164.

27 Chin-Feng L, Ching-Hwa T, Chiung-Hsiang C, et al. Expression of Toll-like receptors in cultured nasal epithelial cells. Acta Otolaryngol 2007;127:395-402.

28 Bonaldi T, Talamo F, Scaffidi P, et al. Monocytic cells hyper-acetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 2003;22:5551-60.

29 Gardella S, Andrei C, Ferrera D, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 2002;3:995-1001.

30 Fiuza C, Bustin M, Talwar S, et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood 2003;101:2652-60.

31 Park JS, Svetkauskaite D, He Q, et al. Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 2004;279:7370-7.

32 Kokkola R, Andersson A, Mullins G, et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. Scand J Immunol 2005;61:1-9.

33 Curtin JF, Liu N, Candolfi M, et al. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. Plos Med 2009;6:e10.

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