FEBS Letters 553 (2003) 190–194 FEBS 27651

Ubiquitin activated tumor necrosis factor receptor associated factor-6 (TRAF6) is recycled via deubiquitination

Liselotte E. Jensen*, Alexander S. Whitehead

Department of Pharmacology and Center for Pharmacogenetics, University of Pennsylvania School of Medicine, 156 Johnson Pavilion, 3620 Hamilton Walk, Philadelphia, PA 19104-6084, USA

Received 29 May 2003; revised 30 July 2003; accepted 19 August 2003

First published online 16 September 2003

Edited by Beat Imhof

Abstract Ubiquitination of intermediates in the interleukin-1 (IL-1) signaling cascade plays an important role in activation and regulation of the pathway. Both IL-1 receptor associated kinase-1 (IRAK1) and inhibitor of nuclear factor κB-α (IκBα) are rapidly ubiquitinated and degraded. Tumor necrosis factor associated factor-6 (TRAF6) is an ubiquitin ligase that is activated by ubiquitination and a signaling intermediate between IRAK1 and IκBα. It is unknown whether activated TRAF6 is subsequently degraded. We show that in liver cells IL-1 stimulates TRAF6 poly-ubiquitination. In less than 1 h levels of non-modified TRAF6 return to levels near those observed prior to activation. TRAF6 cannot be reactivated in cells which have been pretreated with IL-1. This observation correlates with decreased levels of IRAK1 in IL-1 pretreated cells. The re-establishment of non-modified TRAF6 levels following activation does not require de novo protein synthesis, strongly suggesting that TRAF6 is recycled via deubiquitination. This indicates a unique mechanism of regulation of TRAF6 activity.

Key words: Interleukin-1; Tumor necrosis factor associated factor-6; Ubiquitination; Deubiquitination; Desensitization

1. Introduction

Tumor necrosis factor receptor associated factor-6 (TRAF6) is an essential intermediate in multiple signaling pathways regulating such processes as bone resorption and the innate immune response. The latter is initiated by the recognition of pathogen by-products such as lipopolysaccharide, peptidoglycan, CpG-DNA and double stranded RNA by cellular transmembrane Toll-like receptors (TLRs) at the site of injury/infection [1,2] which then leads to interleukin-1 (IL-1) production. IL-1, via its receptor (IL-1R), in turn orchestrates the systemic responses that act in concert with local leukocytes to eliminate the pathogen (reviewed in [3]). Ligand activation of the TLR and IL-1R leads to activation of a common intracellular signaling cascade which initially involves the recruitment of the adapter proteins MyD88 and/or Mal/TIRAP which then recruit IL-1R associated kinase-1 (IRAK1) and IRAK4 to the intracellular domains of the transmembrane receptors (reviewed in [4]). IRAK1 is phosphorylated by IRAK4 [5], an event that may lead to the dissociation of IRAK1 from the membrane complex and allow subsequent interactions with TRAF6. TRAF6 further interacts with transforming growth factor β activated kinase-1 (TAK1) and the TAK1 binding proteins [4]. TAK1 in turn activates the inhibitor of nuclear factor κB (IκB) kinase complex which phosphorylates IκBα and IκBβ, thereby targeting each for ubiquitination and degradation by the proteasome. This permits the liberated NF-κB to translocate to the nucleus where it can initiate transcription from numerous genes important for both the innate and adaptive immune responses.

Ubiquitination, which clearly plays an important role in regulating immune responses (reviewed in [6]), involves the covalent attachment of several subunits of ubiquitin to a given substrate via lysine residues. In the TLR/IL-1 signaling cascade the ubiquitination and subsequent degradation of IκB is the key event that allows NF-κB to be activated. IRAK1 is also ubiquitinated and degraded following engagement of either the TLR or the IL-1R [7]. However, unlike the degradation of IκB which allows activation of NF-κB, degradation of IRAK1 is believed to provide a molecular brake on the signaling cascade by limiting the availability of this factor [7–15].

It has recently been demonstrated that TRAF6 is also ubiquitinated in response to IL-1 and receptor activator of NF-κB ligand (RANKL) [16,17], and that TRAF6 is itself an ubiquitin ligase that is activated by ubiquitination [18]. Although it has been suggested that RANKL induced ubiquitination of TRAF6 targets TRAF6 for degradation [17], the actual fate of TRAF6 following IL-1 activation remains unknown.

Sepsis is a potentially fatal condition and is, in part, characterized by a decreased ability of leukocytes to synthesize pro-inflammatory cytokines. Several studies have examined the consequences of pre-exposure of monocytes/macrophages to bacterial by-products, and have suggested that down-regulation of IRAK1 levels probably plays a role in the desensitization process that may underlie the reduced activity of monocytes/macrophages during sepsis [8–15]. Furthermore, it has been shown that interferon-γ and granulocyte/macrophage colony stimulating factor can prevent desensitization and that this may be due to stabilization of IRAK1 [12]. The liver plays an important part in the acute phase response to infection by synthesizing agents which are essential for eliminating pathogens, e.g. mannose binding lectin, comple-

*Corresponding author. Fax: (1)-215-573 9135.
E-mail address: jensen@pharm.med.upenn.edu (L.E. Jensen).

Abbreviations: IκBα, inhibitor of nuclear factor κB-α; IL-1R, IL-1 receptor; IRAK1, interleukin-1 receptor associated kinase-1; RANKL, receptor activator of NF-κB ligand; TAK1, transforming growth factor β activated kinase-1; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor associated factor-6

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00998-0

ment factors and the major acute phase reactants, C-reactive protein and serum amyloid A protein [19,20]. Although the role of the liver responses in the context of sepsis has been poorly addressed, desensitization of the liver could also play an important role in the pathology of septic shock.

We have investigated the fate of TRAF6 in human hepatoma cells (HepG2) after activation by IL-1 and here show that TRAF6 cycles through ubiquitination and deubiquitination before returning to its pre-activation state. We also show that liver cells become desensitized to IL-1, i.e. TRAF6 cannot be reactivated and IκBα is not degraded, after prior IL-1 treatment.

2. Materials and methods

2.1. Cell cultures

Human hepatoma cells (HepG2) were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (MediaTech, Herndon, VA, USA) supplemented with 10% fetal calf serum (Gemini Bio-Products, Woodland, CA, USA), 1 mM sodium pyruvate, 0.01 mM non-essential amino acids and 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA, USA). All experiments were performed at least three times with similar outcomes.

2.2. Cytokines, antibodies and other agents

IL-1α and IL-1β were obtained from National Cancer Institute, Frederick, MD, USA. Cells were treated with 10 ng/ml unless otherwise stated. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following polyclonal rabbit antibodies were used: anti-IκB-α (FL), anti-IRAK-1 (H-273), anti-TRAF6 (H-274) and anti-ubiquitin (FL-76). Actinomycin D and cycloheximide (Sigma, St. Louis, MO, USA) were used at 5 μg/ml and 50 μg/ml, respectively. The proteasome inhibitors NLVS, MG-132 and ALLN (CNbiosciences, La Jolla, CA, USA) were used at 100 μM.

2.3. Immunoprecipitation and Western blotting

Total proteins were extracted on ice in NP-40 lysis buffer comprising 50 mM HEPES, 150 mM NaCl, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM benzamidine, 50 mM NaF, 1 mM Na_3VO_4, 5 mM *para*-nitrophenyl phosphate, 2 mM dithiothreitol, 10% protease inhibitor cocktail (Sigma), 1% NP-40. Cellular debris was removed by centrifugation at 10 000×*g* for 10 min. Amounts of protein were quantitated using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. To preclude the possibility of relocalization of TRAF6 to relatively insoluble cellular compartments total cellular extracts were also generated in 66% lysis buffer, 2 M urea and 0.85% sodium dodecyl sulfate (SDS). DNA was sheared by passing lysates through a 23G syringe four times. No aggregates could be observed after centrifugation. TRAF6 protein was immunoprecipitated using anti-TRAF6 and protein A agarose (Invitrogen) for 4 h at 4°C with continuous rotation. Immunoprecipitates were washed four times using phosphate buffered saline, 1% NP-40. Proteins were separated in NuPAGE 7% Tris-acetate (TRAF6 and IRAK1) or NuPAGE 4–12% Bis-Tris (IκBα) gels (SDS–polyacrylamide gel electrophoresis (PAGE), Invitrogen) and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Proteins were detected using specific primary rabbit antibodies. Primary antibodies were recognized using horseradish peroxidase conjugated goat anti-rabbit Ig and enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech). Protein bands were quantitated using ImageQuaNT technology (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results and discussion

3.1. TRAF6 is ubiquitinated in response to IL-1 in HepG2 cells

Human HepG2 cells were treated with 10 ng/ml IL-1α and total protein was harvested at various subsequent time points. Levels of TRAF6, IRAK1 and IκBα were examined using Western blotting. TRAF6 migrated as an approximately 60 kDa protein, in agreement with its predicted size based on amino acid sequence. After 5 min, the intensity of the TRAF6 band decreased to approximately 20% that observed at the beginning of the experiment (Fig. 1A), and after 10 min levels were further reduced to 16% of levels present prior to treatment. At the 20 min time point levels of the 60 kDa TRAF6 band had recovered to 72% of that observed in untreated cells and after 30–60 min levels had almost returned to those observed in untreated cells (92–98%, Fig. 1A). No specific bands migrating at higher molecular weight were observed. For comparison IRAK1 and IκBα levels were also examined. IRAK1 was rapidly degraded; within 5 min levels had fallen to approximately 10% of that at 0 min and remained essentially unchanged from this time point through the end of the time course (Fig. 1B). We have recently identified an alternative splice variant of IRAK1, IRAK1b, which is not degraded in response to IL-1 [21]. The low steady-state

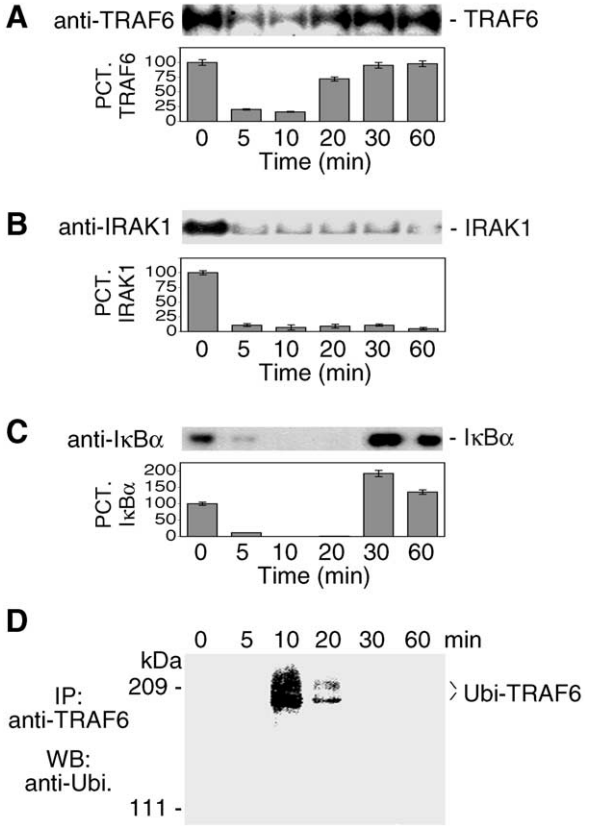

Fig. 1. TRAF6 undergoes ubiquitination in response to IL-1α in HepG2 cells. A–C: Cells were treated with IL-1α and total protein harvested in NP-40 lysis buffer at the indicated time points. Equal amounts of total protein, as determined by Coomassie assays, were separated by SDS–PAGE, and TRAF6 (A), IRAK1 (B) and IκBα (C) detected using Western blotting analysis with specific antibodies. Levels of protein were quantitated and are graphically represented as percentages of those observed at the beginning of the experiment (0 min). D: Cells were treated with IL-1α and total protein was harvested in NP-40 lysis buffer at the indicated time points. TRAF6 was specifically immunoprecipitated using anti-TRAF6. Washed immunoprecipitates were separated by SDS–PAGE and ubiquitinated protein was detected using Western blotting analysis with anti-ubiquitin. Molecular weight size markers are indicated to the left. Positions of ubiquitinated TRAF6 (Ubi-TRAF6) are indicated to the right.

levels of IRAK1 that persist after 5 min may represent this splice variant. IκBα was greatly reduced (90% had disappeared) after 5 min (Fig. 1C) and was completely degraded within 10 min, but reappeared at elevated levels after 30 and 60 min. A nearly two-fold increase was observed after 30 min compared to levels at the beginning of the experiment. After 60 min levels had dropped slightly (1.4-fold increase compared to 0 min). In cells which were treated with only 1 ng/ml IL-1α, the results were similar except that the stages at which protein levels changed were shifted towards later time points (not shown), suggesting a slower processing of proteins. Similar results were obtained using IL-1β at the same respective concentrations (not shown).

It has previously been shown that TRAF6 is ubiquitinated in response to IL-1 in HeLa cells [16]. We therefore examined whether TRAF6 is also ubiquitinated in response to IL-1 in HepG2 cells treated with IL-1α. Total protein was harvested at different time points following IL-1 treatment and TRAF6 was specifically immunoprecipitated. Immunoprecipitated proteins were examined using Western blotting with anti-ubiquitin (Fig. 1D). High levels of ubiquitinated TRAF6 were observed after 10 min, with somewhat lower levels persisting at 20 min. The ubiquitinated TRAF6 migrated with apparent molecular weights of approximately 190 and 210 kDa. Given that the ubiquitin polypeptide is approximately 8.5 kDa, this suggests that TRAF6 was polyubiquitinated with 15–17 ubiquitin units. Although levels of non-modified TRAF6 had already declined by 5 min (Fig. 1A), no high molecular weight ubiquitinated TRAF6 could be detected at this time point (Fig. 1D). However, it is possible that TRAF6 at this stage exists in many different intermediately ubiquitinated forms containing between 1 and 14 ubiquitin subunits and that these intermediates, which differ in increments of 8.5 kDa, do not resolve as discrete bands with intensities that may readily be detected.

The blot containing proteins immunoprecipitated with anti-TRAF6 was reprobed with the same antibody. Again, only the non-modified TRAF6 form was detected (not shown) correlating with the result depicted in Fig. 1A. It is likely that different antibodies from the polyclonal anti-TRAF6 mixture are involved in recognition of native and denatured epitopes, i.e. while some antibodies recognize native epitopes and allow TRAF6 to be immunoprecipitated, others recognize denatured epitopes in Western blotting. If ubiquitin modification precludes recognition of the denatured (but not native) epitopes, immunoprecipitated ubiquitinated TRAF6 may not be directly detectable by Western blotting with anti-TRAF6 but may only be recognized indirectly using antibodies against ubiquitin.

The above observations are in general agreement with previously reported [16] IL-1 induced TRAF6 ubiquitination. However, in contrast to HeLa cells in which only a small proportion of TRAF6 becomes ubiquitinated, and hence no changes in levels of non-modified TRAF6 have been reported [16], in HepG2 cells a substantial amount (approximately 84% based on the decrease in non-modified TRAF6 levels observed in Fig. 1A) of TRAF6 undergoes ubiquitination. Such variation in the magnitude of responses may be due to different numbers of IL-1 receptors and/or intracellular signaling factors expressed by specific cell types. Because of the more dramatic effect upon TRAF6 modification in the HepG2 model this system allows us to address three major questions. (1) Can TRAF6, after pre-modification levels are re-established, be reactivated? (2) Is ubiquitin conjugated TRAF6 eventually degraded? (3) Is the re-constitution of non-modified TRAF6 due to de novo protein synthesis?

3.2. *HepG2 cells become desensitized to IL-1 and TRAF6 cannot be reactivated*

Cells were pretreated with medium only or with IL-1α for 1 or 4 h. Cells were subsequently treated with IL-1α and total protein was harvested after 0, 10, 30 or 60 min. Levels of TRAF6, IRAK1 and IκBα were examined using Western blotting (Fig. 2). In cells pretreated with medium only, a transient decrease in TRAF6 levels was observed after 10 min and levels were returning to pretreatment levels after 30–60 min. IκBα levels were also transiently down-regulated after 10 min and returned after 30–60 min. However, no changes in TRAF6 or IκBα protein levels were observed in cells which had been pretreated with IL-1α for either 1 or 4 h even though levels of these proteins at the end of the pretreatment period (i.e. at the time of the second IL-1α treatment) were similar to those in cells pretreated with medium only (Fig. 2). This suggests that the cells have become desensitized to IL-1. In cells which were pretreated with medium only, IRAK1 was rapidly degraded within 10 min in response to the subsequent IL-1α treatment (Fig. 2). In cells pretreated with IL-1α IRAK1, which was markedly down-regulated relative to the untreated control in response to IL-1α pretreatment, remained at a steady-state suppressed level, i.e. levels were not down-regulated any further following the second IL-1α treatment (Fig. 2).

Several previous studies of monocyte/macrophage desensitization to bacterial by-products have suggested that the down-regulation of IRAK1 plays an important role in the adoption of a desensitized phenotype [8–15]. However, many of these studies have monitored IκB degradation/NF-κB activity and cytokine production, which are, respectively, late

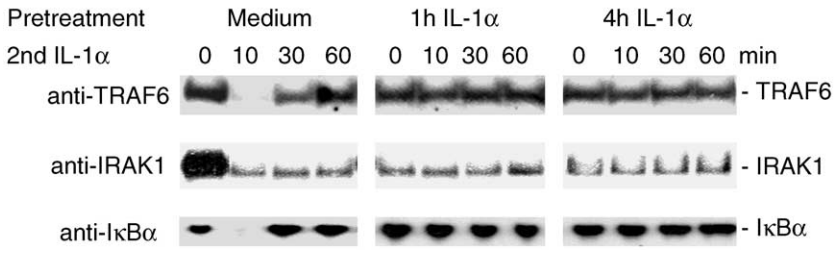

Fig. 2. TRAF6 cannot be reactivated following IL-1α pretreatment. HepG2 cells were pretreated with medium only or with IL-1α for 1 h or 4 h and subsequently treated with IL-1α for 0, 10, 30 and 60 min. Total protein was harvested in NP-40 lysis buffer at each time point and equal amounts of total protein, as determined by Coomassie assays, analyzed using Western blotting.

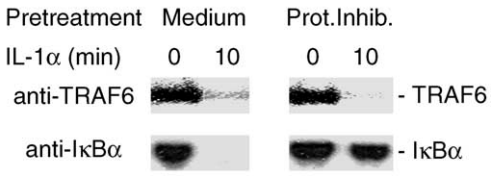

Fig. 3. TRAF6 levels are not affected by the presence of proteasome inhibitors. HepG2 cells were pretreated with medium only or with the proteasome inhibitors NLVS, MG-132 and ALLN (Prot.Inhib.) for 30 min and subsequently treated with IL-1α for 0 or 10 min. Total protein was harvested in NP-40 lysis buffer and equal amounts, as determined by Coomassie assays, were analyzed using Western blotting.

and end-point events in the signaling cascade and hence a consequence of successful signaling. It is therefore a possibility that one or more factors between IRAK1 and the monitored end-point/consequence could also influence desensitization. Our observations reported here that IRAK1 levels are down-regulated following IL-1 treatment, and that this down-regulation correlates with an inability to activate the immediate downstream signaling component TRAF6, further strengthen the previous hypothesis that IRAK1 becomes a limiting factor for the signaling cascade. The data also demonstrate, to our knowledge for the first time, that liver cells (here HepG2 cells) become desensitized to IL-1 during inflammation. Such desensitization may play an important role during sepsis since the liver is the major site of synthesis of several circulating proteins (e.g. mannose binding lectin and complement factors) which are directly involved in the immune responses directed at eliminating pathogens [19,20].

3.3. TRAF6 is recycled via deubiquitination

Ubiquitination of IRAK1 and IκBα targets each of these proteins for degradation. While levels of IRAK1 remain suppressed for an extended period of time [8–15,21], IκBα rapidly reappears in the cytoplasm. The latter is due to de novo NF-κB driven gene transcription and protein synthesis (reviewed in [22]). Although it has previously been reported that TRAF6 is ubiquitinated following IL-1 stimulation [16], it has not been determined if TRAF6 is subsequently degraded. To examine whether inhibition of the proteasome complex would affect TRAF6 modification, cells were pretreated with medium only or the proteasome inhibitors NLVS, MG-132 and ALLN for 30 min and subsequently treated with IL-1. Total protein lysates were harvested and levels of TRAF6 and IκBα examined. Although degradation of IκBα was inhibited, demonstrating activity of the proteasome inhibitors, no effect upon TRAF6 was observed (Fig. 3).

Since the reappearance of TRAF6 (Fig. 1A) in its non-modified state 30 min after IL-1 treatment resembles that of IκBα (Fig. 1C) we tested whether inhibition of de novo gene transcription could prevent the reconstitution of non-modified TRAF6. Cells were pretreated with medium only or actinomycin D for 1 h after which cells were treated with IL-1α. Although the actinomycin D pretreatment completely abolished the reappearance of IκBα, this pretreatment had no effect upon the regeneration of non-modified TRAF6 (Fig. 4). Nevertheless, it remained a possibility that TRAF6 could be reconstituted by translation from a pre-existing pool of TRAF6 mRNA. We therefore performed a similar experiment in which cells were pretreated with cycloheximide to inhibit de novo protein synthesis. Again the reappearance of IκBα was completely prevented (Fig. 4). However, TRAF6 reappeared after 30 min irrespective of whether cells had been pretreated with medium only or with cycloheximide (Fig. 4), thereby precluding the possibility that TRAF6 is synthesized de novo. These observations led us to conclude that the non-modified TRAF6 levels observed after 30–60 min of IL-1α treatment could only be derived from the pool of TRAF6 which was seen to be ubiquitinated at the earlier 10 and 20 min time points (Fig. 1D). Such a recycling process would require removal of the polyubiquitin chain by deubiquitinating enzymes of which 90 currently are known (reviewed in [23]). It has previously been shown that TRAF6 is polyubiquitinated via a unique chain formation at the lysine residue 63 (K63) in the ubiquitin polypeptide sequence rather than K48 [18]. It is possible that this unique polyubiquitin chain targets TRAF6 for recycling instead of degradation. Given that TRAF6 needs to be ubiquitinated in order to be activated, it appears most likely that deubiquitination inactivates TRAF6.

4. Conclusion

TRAF6 has recently been demonstrated to be a ubiquitin ligase which is itself activated by ubiquitination in response to IL-1. Several other components in the IL-1 signaling cascade are ubiquitinated in response to IL-1 and are consequently targeted for degradation. Here we have shown that TRAF6 distinguishes itself by being recycled via deubiquitination, hence establishing a novel regulatory mechanism of IL-1 signaling. Desensitization of liver cells may play an important role during sepsis. We have further shown that TRAF6 cannot be reactivated by IL-1 in cells which have been pretreated with IL-1, and that this lack of reactivation appears to correlate with decreased levels of IRAK1. Further studies of the mechanisms of desensitization of both leukocytes and hepatocytes may prove valuable in developing therapies against sepsis.

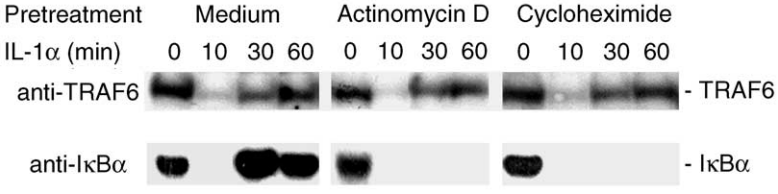

Fig. 4. Non-modified TRAF6 reappears independently of de novo gene transcription or protein synthesis. HepG2 cells were pretreated with medium only, actinomycin D or cycloheximide. Cells were subsequently treated with IL-1α and total protein harvested in NP-40 lysis buffer at the time points indicated. Proteins were detected using Western blotting.

References

[1] Modlin, R.L. (2002) Ann. Allergy Asthma Immunol. 88, 543–547.
[2] Triantafilou, M. and Triantafilou, K. (2002) Trends Immunol. 23, 301–304.
[3] Dinarello, C.A. (1996) Blood 87, 2095–2147.
[4] Martin, M.U. and Wesche, H. (2002) Biochim. Biophys. Acta 1592, 265–280.
[5] Li, S., Strelow, A., Fontana, E.J. and Wesche, H. (2002) Proc. Natl. Acad. Sci. USA 99, 5567–5572.
[6] Ben-Neriah, Y. (2002) Nat. Immunol. 3, 20–26.
[7] Yamin, T.T. and Miller, D.K. (1997) J. Biol. Chem. 272, 21540–21547.
[8] Swantek, J.L., Tsen, M.F., Cobb, M.H. and Thomas, J.A. (2000) J. Immunol. 164, 4301–4306.
[9] Li, L., Cousart, S., Hu, J. and McCall, C.E. (2000) J. Biol. Chem. 275, 23340–23345.
[10] Hu, J., Jacinto, R., McCall, C. and Li, L. (2002) J. Immunol. 168, 3910–3914.
[11] Jacinto, R., Hartung, T., McCall, C. and Li, L. (2002) J. Immunol. 168, 6136–6141.
[12] Adib-Conquy, M. and Cavaillon, J.M. (2002) J. Biol. Chem. 277, 27927–27934.
[13] Yeo, S.J., Yoon, J.G., Hong, S.C. and Yi, A.K. (2003) J. Immunol. 170, 1052–1061.
[14] Sato, S., Takeuchi, O., Fujita, T., Tomizawa, H., Takeda, K. and Akira, S. (2002) Int. Immunol. 14, 783–791.
[15] Yamashina, S., Wheeler, M.D., Rusyn, I., Ikejima, K., Sato, N. and Thurman, R.G. (2000) Biochem. Biophys. Res. Commun. 277, 686–690.
[16] Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J. and Chen, Z.J. (2001) Nature 412, 346–351.
[17] Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K. and Taniguchi, T. (2000) Nature 408, 600–605.
[18] Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. and Chen, Z.J. (2000) Cell 103, 351–361.
[19] Baumann, H. and Gauldie, J. (1994) Immunol. Today 15, 74–80.
[20] Steel, D.M. and Whitehead, A.S. (1994) Immunol. Today 15, 81–88.
[21] Jensen, L.E. and Whitehead, A.S. (2001) J. Biol. Chem. 276, 29037–29044.
[22] Jensen, L.E. and Whitehead, A.S. (1998) Biochem. J. 334, 489–503.
[23] Chung, C.H. and Baek, S.H. (1999) Biochem. Biophys. Res. Commun. 266, 633–640.