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# Interleukin-1 receptor-associated kinase and TRAF-6 mediate the transcriptional regulation of interleukin-2 by interleukin-1 via NFκB but unlike interleukin-1 are unable to stabilise interleukin-2 mRNA

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**Abstract**

Interleukin-1 receptor-associated kinase, IRAK, has been shown to activate NFκB in response to interleukin-1. We have explored the involvement of IRAK in regulation of the interleukin-2 gene in the murine thymoma cell line EL4.NOB-1 by examining its effect on interleukin-2 promoter-linked reporter gene expression, interleukin-2 gene transcription and interleukin-2 protein production. Cells transfected with IRAK displayed high levels of phosphorylated IRAK, increased interleukin-2 promoter-linked reporter gene expression (which was dependent on NFκB) and interleukin-2 gene transcription. IRAK was unable, however, to increase interleukin-2 protein production. Overexpression of TRAF-6 induced similar responses and again failed to increase interleukin-2 protein production. A dominant negative TRAF-6 inhibited reporter gene expression and interleukin-2 protein production in response to both interleukin-1 and IRAK transfection. Interleukin-1 treatment and IRAK or TRAF-6 transfection increased interleukin-2 mRNA production. Only interleukin-1 treatment stabilised the induced transcripts with 50% being detectable at 20 h post induction. The interleukin-2 mRNA induced in IRAK- or TRAF-6-transfected cells was depleted by $>90\%$ at 6 h post induction. These data implicate IRAK and TRAF-6 in transcriptional regulation of interleukin-2 gene expression via NFκB, and provide direct evidence that IRAK lies upstream from TRAF-6. Neither IRAK nor TRAF-6 participates in stabilisation of interleukin-2 mRNA which is required for interleukin-2 protein production. 

*Keywords:* Interleukin-1; Interleukin-2; Interleukin-1 receptor-associated kinase; Tumor necrosis factor receptor-associated factor-6; Regulation; Transcription

Abbreviations: CAT, chloramphenicol acetyl transferase; CIP, calf intestinal alkaline phosphatase; hTLR, human Toll-like receptor; IKK, IκB kinase; IL-1, interleukin-1; IL-1RI, interleukin-1 type I receptor; IL-1RII, interleukin-1 type II receptor; IL-1RAcp, interleukin-1 receptor accessory protein; IL-1Rrp, interleukin-1 receptor-related protein; IL-2, interleukin-2; IL-18, interleukin-18; IRAK, interleukin-1 receptor-associated kinase; JNK, Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; NIK, NFκB-inducing kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; TRAF, tumor necrosis factor receptor-associated factor; TRAF-6Δ, dominant negative TRAF-6

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## 1. Introduction

IL-1 is a principal cytokine responsible for the induction of mediators which orchestrate the immune and inflammatory responses. The potent proinflammatory effects induced by IL-1 are co-ordinated by the increased expression of a variety of IL-1-inducible genes. Following engagement of IL-1 with its receptor, signals are transduced intracellularly causing transcription factor activation and enhanced gene expression. Two distinct IL-1 cell surface receptors exist, an 80 kDa type I receptor (IL-1RI) [1] and a 68 kDa type II receptor (IL-1RII) [2]. The IL-1 signal is transduced exclusively through IL-1RI [3]. IL-1RII acts as a regulatory decoy molecule [4]. The existing model of IL-1 receptor interaction involves the engagement by IL-1 of its type I receptor with subsequent heterodimer formation between IL-1RI and the IL-1 receptor accessory protein (IL-1RAcp). This receptor complex transduces the IL-1 signal inside the cell.

An ubiquitous signal triggered in response to IL-1 is activation of the transcription factor NFκB. Recent advances have identified two signal transducers involved in this process: an IL-1 receptor-associated kinase, IRAK [5–7], and TRAF-6, a member of the TNF receptor-associated factor family [8]. Originally detected as a serine/threonine protein kinase activity which could be coprecipitated with IL-1RI from IL-1-stimulated T cells [9], IRAK was subsequently identified and shown to be required for IL-1-induced NFκB activation [7]. TRAF proteins are primarily involved in TNF receptor-mediated NFκB activation [9–12]. TRAF-6 is structurally and functionally related to the five other characterised TRAF proteins. However, it participates in IL-1- rather than TNF-induced NFκB activation [8].

The current model of IL-1-induced NFκB activation involves signal transduction through a multiprotein complex in which the cytosolic protein MyD88, a member of the IL-1 receptor family, acts as an adaptor coupling the receptor complex to IRAK and IRAK-2 [13–17]. It has been proposed that the IL-1 signal is relayed from IRAK and IRAK-2 through TRAF-6 to the kinase complex TAB1/TAK1 which then activates NFκB-inducing kinase (NIK), the upstream regulator of the IκB kinases, IKK1 and IKK2 [18–23]. IκB becomes phosphorylated and releases NFκB which translocates to the nucleus and induces target gene expression.

In this study, we have examined in detail the role played by IRAK and TRAF-6 in the regulation of the IL-2 gene in the murine thymoma cell line EL4.NOB-1. Several responses are induced in this cell line by IL-1, including activation of transcription factors, MAP kinase cascades and the production of IL-2. We confirm that NFκB activation by IL-1 is regulated by IRAK and TRAF-6 in these cells and show that both proteins are critical for IL-2 promoter function and IL-2 gene transcription. The effects of IRAK and TRAF-6 on IL-2 production are restricted to transcription, however, and neither is able to stabilise the mRNA transcript for IL-2. Such stabilisation must therefore be required for IL-2 protein production and is regulated in response to IL-1. The results imply that IRAK and TRAF-6 only participate in the regulation of IL-2 gene transcription by IL-1. Additional IL-1-induced signals including IL-2 mRNA stabilisation are required, without which no IL-2 protein production will occur.

## 2. Materials and methods

### 2.1. Materials

The EL4.NOB-1 cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). RPMI 1640 medium and foetal calf serum were from HyClone Europe (Cramlington, UK). Penicillin/streptomycin and L-glutamine (2 mM final concentration) were purchased from Life Technologies. Human recombinant IL-1α was a gift from Prof. J. Saklatvala (Kennedy Institute of Rheumatology, London, UK). [$^{14}$C]Chloramphenicol (54 mCi/mmol) and [α-$^{32}$P]dATP (triethylammonium salt, 3000 Ci/mmol) were from Amersham International (Aylesbury, UK). Restriction enzymes, DNA modifying enzymes, dNTPs, CIP and ECL LumiGlo reagent and peroxide were from NEB (Hertfordshire, UK). Agarose, actinomycin D ($C_1$), Rapid DNA ligation, Titan RT-PCR and First strand cDNA synthesis kits were purchased from Boehringer Mannheim (East Sussex, UK). Oligonucleotide primers

were from MWG Biotech (Milton Keynes, UK). Prime-a-Gene kit was from Promega (Madison, WI, USA). The $(NF\kappa B)_5$-linked CAT reporter plasmid was from Dr. T. Bird (Immunex, Seattle, WA, USA), the IL-2 promoter-CAT plasmid was a gift from Dr. W. Falk (University of Regensburg, Germany). Anti-IRAK antiserum, plasmid pRK5 (empty vector) and the IRAK, TRAF-6 and TRAF-6Δ expression plasmids were generously supplied by Dr. D.V. Goeddel (Tularik, San Francisco, CA, USA). Purified murine anti-IL-2, recombinant murine IL-2 and biotinylated murine anti-IL-2 were supplied by Pharmingen (San Diego, CA, USA). CytoXpress quantitative PCR kit for mouse IL-2 was from BioSource International (Camarillo, CA, USA). All other materials were from Sigma (Poole, UK).

### 2.2. Cell culture and transfection

EL4.NOB-1 cells were cultured in RPMI 1640 containing 10% foetal calf serum, 2 mM L-glutamine, penicillin and streptomycin and were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. The cells were transfected with indicated amounts of expression plasmid and 10 μg of reporter plasmid. In dose-response experiments the concentration of DNA was maintained constant by supplementation with pRK5. Briefly, $1.4\times10^7$ cells were incubated with plasmid DNA in TBS (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.6 mM $NaH_2PO_4$) containing 3 μg DEAE dextran and 50 μg chloroquine for 30 min. The cells were washed in RPMI 1640 containing 5 U/ml heparin. Following two additional washes with medium, cells were left to recover for 16 h in medium supplemented with 20% FCS. Aliquots of cells were treated for further time periods with human recombinant IL-1α (10 ng/ml) or actinomycin D (1 mM) before harvesting cell extracts, RNA or culture supernatants.

### 2.3. Western blotting

Cells ($5\times10^6$) were washed with 1 ml ice cold PBS and suspended in 100 μl extraction buffer (1% (v/v) Triton X-100, 20 mM Tris acetate, pH 7, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM tetrasodium pyrophosphate, 270 mM sucrose, 10 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride, 2 μg/ml aprotinin and 1 μg/ml leupeptin). Following a 15 min incubation on ice the samples were centrifuged at $14\,000\times g$ for 10 min at 4°C. The protein concentration of the supernatants was determined [24] and aliquots containing 2.5 μg of protein were left untreated or incubated with 1 U CIP for 30 min at 37°C. Samples were mixed with 2×Laemmli sample buffer, boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose filters and these were blocked in PBS, 0.05% Tween containing 5% (w/v) non-fat milk. IRAK expression was detected using rabbit polyclonal anti-IRAK antibodies (1:1000). The filters were washed and incubated with protein A peroxidase (1:1000). Signals were developed using enhanced chemiluminescence (ECL).

### 2.4. CAT assays

Cells were harvested at $150\times g$ for 10 min and washed twice in 1 ml PBS, then pelleted by centrifugation at $14\,000\times g$ for 5 min. Cell pellets were resuspended in 0.25 M Tris-HCl, pH 8 and lysed by three cycles of freezing in liquid $N_2$ and thawing at 37°C. Protein concentration in each cell extract was determined by the Bradford method [24]. Extracts containing 20 μg protein were incubated at 37°C overnight in buffer containing 1 mM acetyl-CoA and 0.3 mCi [$^{14}$C]chloramphenicol. The reactions were stopped by addition of 350 μl ethyl acetate, vortexed for 30 s and the phases separated by centrifugation at $14\,000\times g$ for 1 min. The upper phase was transferred to a fresh tube, vacuum dried and suspended in 12 ml ethyl acetate. Acetylated and non-acetylated complexes were resolved by silica gel TLC in 19:1 (v/v) chloroform:methanol. The separated products were quantified using image analysis (InstantImager, Packard Instrument, Meriden, CT, USA).

### 2.5. Measurement of IL-2 production

IL-2 was measured from total cell extracts (see Section 2.3) or the supernatants of control and transfected cells ($1\times10^6$) which had been left untreated or stimulated with IL-1α (10 ng/ml) for 24 h. It was not

necessary to select stable transfectants as transfection efficiencies of $>80\%$ were routinely obtained. IL-2 was measured by ELISA. Microtitre plates were coated with anti-mouse IL-2 (4 μg/ml in 0.1 M $NaHCO_3$, pH 8.2). Additional binding sites were blocked with PBS/10% FCS and the plates washed with PBS containing 1% Tween. Standards and samples, 100 μl, were added and following washes were incubated with anti-IL-2 (2 μg/ml in PBS/10% FCS) and avidin peroxidase (2.5 μg/ml in PBS/10% FCS). Signals were detected following addition of substrate (300 ng/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) in 100 mM citric acid, pH 4.35, containing 0.03% $H_2O_2$) by measuring absorbances at 405 nm.

### 2.6. RNA extraction and analysis

Total RNA was isolated from $5\times10^6$/ml control or transfected cells, which had been left untreated, were stimulated with IL-1α (10 ng/ml) for 6 or 24 h or to which actinomycin D (1 μM) was added for 3, 6 or 24 h, using TRI reagent according to the manufacturer's instructions. All RNA samples were treated with 50 U RNase-free DNase for 30 min at 37°C. Initially RT-PCR reactions (Titan, Boehringer Mannheim) were performed as described in the manufacturer's protocol using oligonucleotide primers 5′-AAGCTCCACTTCAAGCTCTACAGCG-3′ (forward) and 5′-TTGACAGAAGGCTATCCATCTCCTC-3′ (reverse) to amplify a 412 bp product from the IL-2 transcript and 5′-TTCCACGAGACACTGTACCAGAAGG-3′ (forward) and 5′-ACCATGTTGGGCTTCAGCAATG-3′ (reverse) to amplify a 464 bp product from the control aldolase transcript. Thermocycling conditions were as follows: 50°C for 30 min, 94°C for 2 min; ten cycles of 94°C for 30s, 55°C for 30 s and 68°C for 1 min; 25 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 1 min plus 5 s/cycle and were carried out in a thermocycler from MJ Research (Watertown, MA, USA). A final extension step of 68°C for 7 min was followed by resolution of the products on a 1.5% TAE agarose gel containing 0.5 μg/ml ethidium bromide. The relative amount of IL-2 and aldolase transcript in each sample was determined semi-quantitatively following analysis by UVP transillumination densitometry scanning and Gelworks 1D Advanced software. RNA degradation was estimated by quantitation of RT-PCR products blotted onto nylon membranes and hybridised to α-$^{32}$P random primer labelled IL-2 or aldolase cDNAs. The signals were quantitated using image analysis (InstantImager, Packard Instrument) and normalised for the amount of aldolase mRNA present.

For competitive IL-2 PCR reactions, 1 μg RNA was reverse transcribed into cDNA according to the manufacturer's instructions (Boehringer Mannheim). The integrity of RNA extraction and cDNA synthesis was verified by measuring the amount of aldolase cDNA in each sample. Conditions for quantitative competitive PCR were determined by carrying out titration curves for IL-2 cDNAs and internal calibration standards (ICS) in PCR reactions as described in the manufacturer's protocol (BioSource International). This is a recently developed method which allows quantitative measurements of murine IL-2 mRNA. The ICS template contained PCR primer binding sites identical to the IL-2 cDNA which generated a 327 bp ICS product following PCR amplification. This was easily distinguishable by agarose gel electrophoresis from the 277 bp IL-2 PCR product. Microplate detection analysis using ICS- and IL-2-specific capture oligonucleotides bound to microtitre plate wells was carried out to determine the amounts of biotin-labelled ICS and IL-2 amplicons. In order to acquire quantitative results over a broad range, denatured amplicons were analysed by serial dilutions. PCR products were detected and quantified by addition of streptavidin-HRP followed by substrate and measurement of optical density (OD) at 450 nm. The number of copies of IL-2 cDNA in each sample was calculated from the ratio of the total IL-2 OD to the total ICS OD and the input copy of the ICS.

RT-PCR was used to confirm expression of the transfected cDNAs using the IRAK-specific primers 5′-AGCAGAGCAGCTGCAGAGA-3′ (forward) and 5′-GGTGAAGACATCAGCTCT-3′ (reverse) to amplify a 427 bp fragment of the IRAK transcript, and the TRAF-6-specific primers 5′-CATGCGCTTGCACCTTCA-3′ (forward) and 5′-CTATACCCCTGCATCAGT-3′ (reverse) to amplify a 399 bp fragment of the TRAF-6 or TRAF-6Δ transcripts.

## 3. Results

### 3.1. IRAK and TRAF-6 induce NFκB-linked reporter gene expression in EL4.NOB-1 cells

IRAK activity has previously been detected in EL4 and 293 cells following stimulation with IL-1 [6,7]. In 293 cells coexpressing IL-1RI and IL-1RAcp two species of IRAK can be detected by immunoblotting, an 80 kDa unphosphorylated inactive form and a 100 kDa active phosphorylated form [15]. We examined IRAK expression in EL4.NOB-1 cells transfected with a human IRAK cDNA by Western blotting using anti-human IRAK antibodies. This was carried out on extracts from control and IL-1-treated cells (Fig. 1A). The transfected cells expressed detectable levels of IRAK. Both in the absence and presence of IL-1 stimulation, most of the IRAK was detected at a molecular mass of 100 kDa (Fig. 1A, lanes 1 and 2). This corresponded to phosphorylated IRAK since treatment of extracts from control and IL-1-stimulated cells with calf intestinal phosphatase converted the 100 kDa form into an 80 kDa protein, whose molecular mass corresponded to dephosphorylated IRAK (Fig. 1A, lanes 3 and 4). A band of 75 kDa was detected but this was seen in non-transfected cells and was of unknown identity (not shown).

We next tested whether IRAK overexpression would increase the expression of an NFκB-linked reporter gene in EL4.NOB-1 cells. As shown in Fig. 1B, in control cells the NFκB-CAT reporter gene was activated greater than 4-fold over control following stimulation with IL-1. Transfection with the IRAK cDNA induced reporter gene expression in the absence of IL-1, with 10 μg of expression plasmid inducing a maximum effect of 5-fold over control. Addition of IL-1 to the cells overexpressing IRAK showed only a marginal increase over untreated transfected cells indicating that IRAK expression alone optimally activated NFκB. This was consistent with our observation shown in Fig. 1A, where overexpression of IRAK results in its activation, as indicated by phosphorylation. Even doses of IRAK cDNA as low as 0.5 μg induced optimal responses. At a dose of 20 μg the activating effects induced by IRAK overexpression were abolished both in the absence and presence of IL-1 stimulation because of

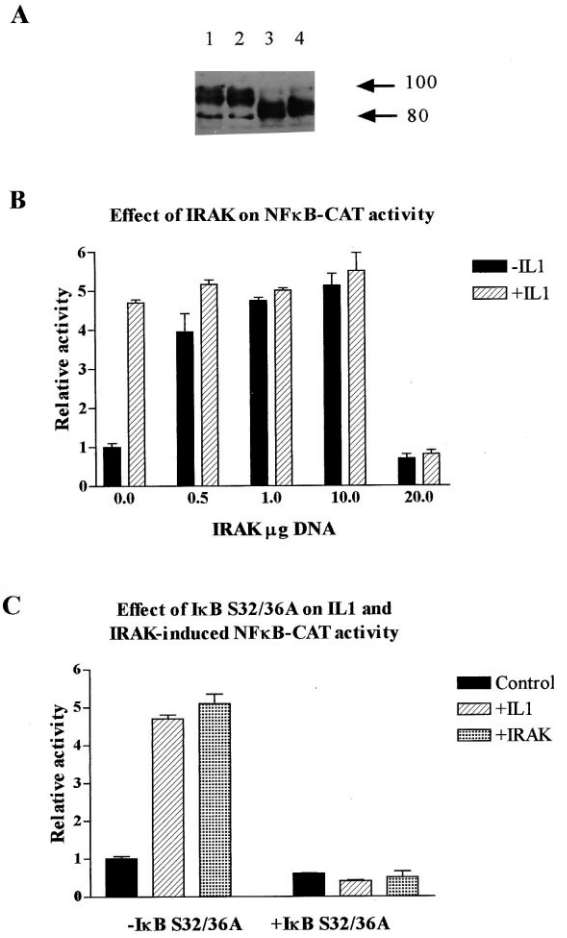


Fig. 1. IRAK overexpression induces NFκB-linked gene expression. (A) Detection of IRAK in transfected cells. Protein extracts (2.5 μg) from control (lanes 1 and 3) and IL-1-stimulated cells (10 ng/ml) (lanes 2 and 4) which had been transfected with an IRAK expression plasmid were left untreated (lanes 1 and 2) or incubated with CIP (1 U) at 37°C for 30 min (lanes 3 and 4). Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with antiserum to IRAK. Reactive proteins of 80 kDa and 100 kDa were detected by ECL. Molecular masses are indicated in kilodaltons. (B,C) EL4.NOB-1 cells were transfected with an $(NF\kappa B)_5$-linked CAT reporter plasmid (10 μg) along with IRAK (B) and/or IκB S32A/S36A mutant (C) expression plasmids as indicated. Following recovery (16 h) cells ($1\times10^6$ per sample) were stimulated with IL-1 (10 ng/ml) for 24 h or left untreated as shown. The CAT reporter gene activity of samples containing 20 μg protein was determined by measuring % acetylation of an acetyl-CoA substrate by [$^{14}$C]chloramphenicol. Acetylated complexes were resolved by silica gel TLC and quantified using image analysis. The data represent the mean ± S.D. from triplicate determinations and are expressed as relative activities compared to individual controls. Similar results were obtained in at least two further experiments.

toxicity. This was not as a result of DNA toxicity since control samples were unaffected at similar DNA concentrations. Therefore it appears that when expressed at high concentrations IRAK can induce killing of EL4.NOB-1 cells.

We also confirmed that the reporter construct being used was driven by NFκB by cotransfecting cells with a gene encoding an IκBα mutant in which serines at positions 32 and 36 are both mutated to alanines (IκB S32/36A). The resulting mutant protein complexes with NFκB, preventing it activation, as described in [25]. As can be seen in Fig. 1C, expression of this mutant prevented both IL-1- and IRAK-induced NFκB-linked reporter gene expression, confirming that the effect of IL-1 and IRAK on the construct were specific for NFκB.

### 3.2. Expression of IRAK activates the IL-2 promoter

We next examined the effects of IRAK on an IL-1-responsive gene promoter which can be activated in EL4.NOB-1 cells, the murine IL-2 promoter up to −273. This carries response elements for NFκB, AP1 and NFAT, amongst others [26].

As shown in Fig. 2A expression of IRAK induced a 4.5-fold activation of the reporter gene. The control empty vector, pRK5, had no effect. Following treatment with IL-1, transcription from the IL-2 promoter was stimulated 2.5-fold. Addition of IL-1 to cells expressing IRAK did not enhance the 4.5-fold potentiation seen following IRAK expression in the absence of IL-1, again indicating that overexpressed IRAK is optimally active. Cotransfection of the S32A/S36A mutant of IκB completely blocked the increase in reporter gene expression confirming the critical dependence of this reporter construct on NFκB for responses induced by IL-1 and IRAK.

### 3.3. IRAK does not increase IL-2 protein production

We next assessed whether expression of IRAK would also increase IL-2 protein production. Typically, we achieved 80% transfection efficiency in EL4.NOB-1 (as measured by FACS analysis of cells expressing a rat CD2 construct; not shown). It is therefore possible to measure IL-2 protein production by ELISA in transfected cells. Intriguingly, we found no effect on IL-2 production upon transfection

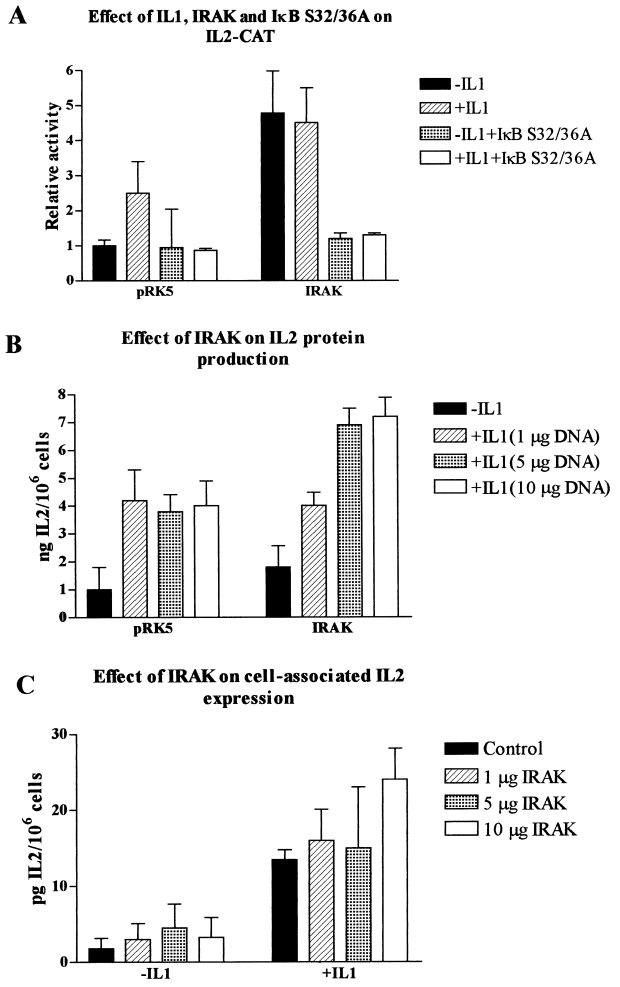


Fig. 2. Effect of IRAK on IL-2 promoter activation and IL-2 protein production. (A) EL4.NOB-1 cells were transfected with an IL-2 promoter-linked CAT reporter plasmid (10 μg), pRK5 (empty vector), IRAK and/or IκB S32A/S36A expression plasmids (10 μg) as indicated. Following recovery (16 h) cells ($1\times10^6$ per sample) were left untreated or stimulated with IL-1 (10 ng/ml) for 24 h. The CAT reporter gene activity of samples containing 20 μg protein was determined by measuring % acetylation of an acetyl-CoA substrate by [$^{14}$C]chloramphenicol. (B) Supernatants were removed or (C) cell extracts were isolated, these were assayed for IL-2 by ELISA as described. The data represent the mean ± S.D. from triplicate determinations and are expressed as relative activities compared to individual controls (A), IL-2 production in ng/$10^6$ cells (B) or pg/$10^6$ cells (C). Similar results were obtained in at least two further experiments.

of cells with IRAK, as shown for 10 μg of IRAK-encoding plasmid in Fig. 2B. IL-1 increased IL-2 production 4-fold in the cells. This response was unaffected by transfection of the cells with 1, 5 or 10 μg

pRK5 control plasmid. Addition of IL-1 to IRAK-expressing cells caused a dose-dependent potentiation in these responses however, resulting in a maximum 7-fold increase in IL-2 production. These results suggested that additional IL-1 signals were required to induce IL-2 protein production with IRAK participating in the pathway by regulating NFκB.

In order to address the identity of such signals we first examined secretion of IL-2, as it was possible that IRAK increased IL-2 protein production, but that an additional signal was required to induce secretion. We therefore measured the total cellular levels of IL-2 produced from each population of cells. IRAK expression did not lead to an increase in cell-associated IL-2, as shown in Fig. 2C. Following treatment with IL-1 (10 ng/ml for 24 h) the levels of IL-2 detectable in each cell lysate were elevated compared to untreated samples, and a potentiation in the response was observed in cells transfected with 10 μg of IRAK-encoding plasmid. This result implied that IRAK did not induce an increase in IL-2 protein while failing to induce IL-2 secretion.

### 3.4. Effect of TRAF-6 on IL-2 gene expression

The results with IRAK implied that it was capable of inducing IL-2 gene expression but was unable to increase IL-2 protein production. Since TRAF-6 occurs downstream from IRAK on the pathway to NFκB we next determined whether a similar phenomenon would apply to TRAF-6. Transfection of cells with TRAF-6 cDNA induced NFκB-linked reporter gene expression in a dose-dependent manner (Fig. 3A). The effect of 0.5 and 1 μg of TRAF-6-encoding plasmid was potentiated by IL-1 to an optimum level of 6-fold over control. Furthermore, 20 μg of TRAF-6 was not toxic to the cells, unlike that observed with IRAK at this plasmid concentration, implying that the toxicity observed with IRAK was not due to a non-specific effect of recombinant protein overexpression.

TRAF-6 expression also increased IL-2 promoter-linked reporter gene expression in a similar manner to IL-1, 2.5-fold over control levels (Fig. 3B). This was dependent on NFκB as the IκBα mutant again inhibited this response (Fig. 3B). Similar to IRAK, TRAF-6 expression was unable to induce IL-2 protein production (Fig. 3C). The effect of IL-1 was

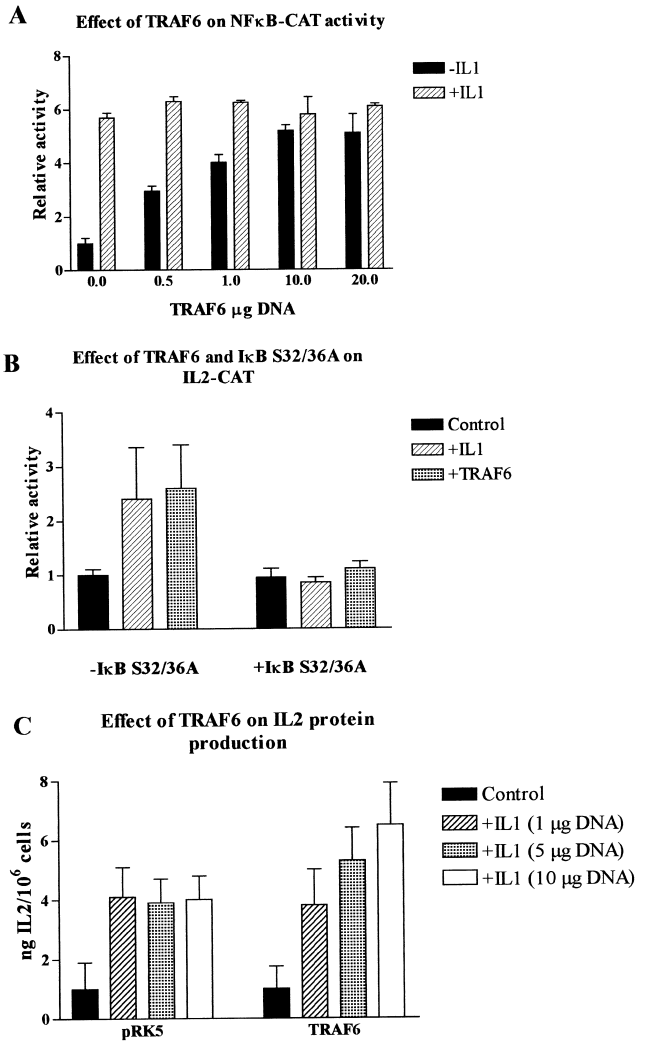


Fig. 3. Effect of TRAF-6 on NFκB- and IL-2 promoter-linked reporter gene expression and IL-2 protein production. EL4.-NOB-1 cells were transfected with pRK5, TRAF-6 and IκB S32A/S36A expression plasmids and NFκB- or IL-2 promoter-linked CAT reporter plasmids as indicated. Following recovery (16 h) cells ($1\times10^6$ per sample) were stimulated with IL-1 (10 ng/ml) or left untreated. The NFκB-CAT (A) or IL-2 promoter-linked CAT (B) gene activity of samples containing 20 μg protein was determined by measuring % acetylation of an acetyl-CoA substrate by [$^{14}$C]chloramphenicol. Supernatants were removed (C) and were assayed for IL-2 by ELISA as described. The data represent the mean ± S.D. from triplicate determinations and are expressed as relative activities compared to individual controls (A,B) or IL-2 production in ng/$10^6$ cells (C). Similar results were obtained in at least two further experiments.

however potentiated in TRAF-6-transfected cells, particularly at 10 μg of TRAF-6 cDNA, where over 7 ng/$10^6$ cells IL-2 was induced, compared to

4 ng/$10^6$ cells in IL-1-treated cells transfected with the empty vector (Fig. 3C).

We also tested the effect of TRAF-6Δ, a dominant negative mutant of TRAF-6 in which the entire zinc-binding domain of TRAF-6 is deleted, on IL-1 and IRAK responses. This has been shown to suppress IL-1-stimulated NFκB activation in 293 cells [8,27]. In response to IRAK transfection the relative activities of NFκB- and IL-2 promoter-linked reporter gene expression were inhibited 60.3 ± 4.1% and 87 ± 3.3% respectively by TRAF-6Δ expression, providing direct evidence that TRAF-6 lies downstream from IRAK. In addition, expression of TRAF-6Δ blocked all of the responses to IL-1 in EL4.NOB-1 cells. IL-1-induced NFκB-CAT and IL-2 promoter-CAT activities were inhibited 64 ± 3.9% and 74 ± 0.4%, respectively. Inhibition of 50 ± 8.8% was achieved in IL-2 protein production by TRAF-6Δ. This implicates TRAF-6 as an important signal transducer of the IL-1 pathway leading to IL-2 gene transcription and protein production in T cells.

Overall, these results therefore implied that similar to IRAK, TRAF-6 increases IL-2 gene transcription, but is unable to induce subsequent events required for IL-2 protein production.

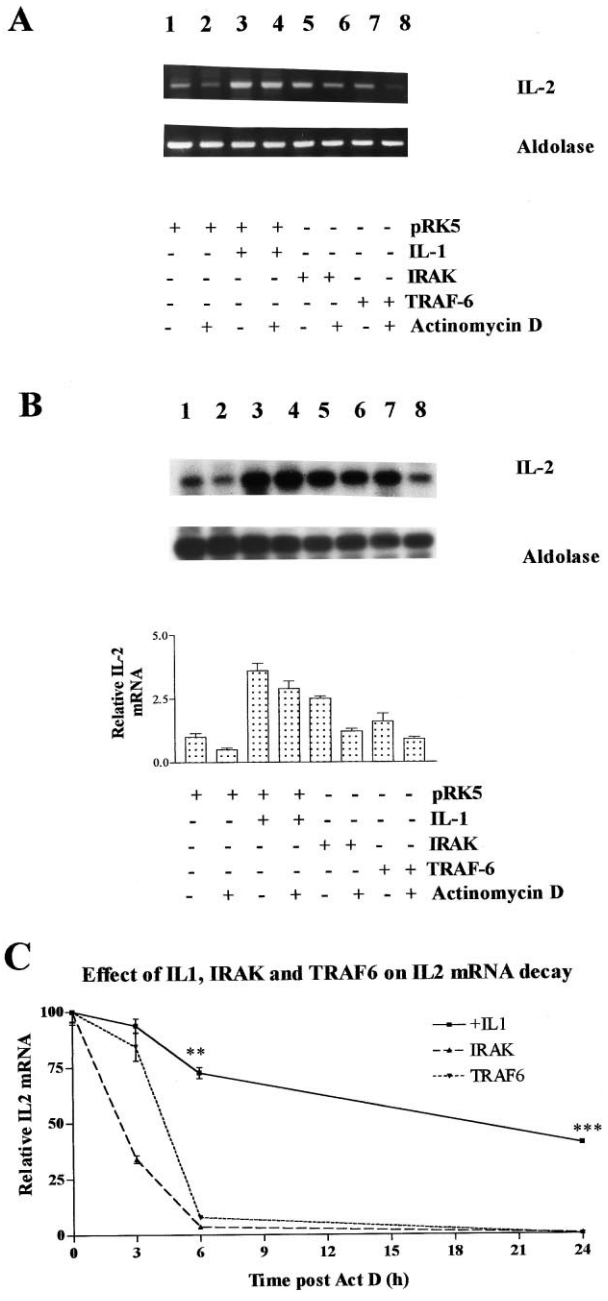


Fig. 4. Effect of IL-1, IRAK and TRAF-6 on IL-2 mRNA accumulation and decay. EL4.NOB-1 cells were transfected with pRK5, IRAK or TRAF-6 expression plasmids (10 μg). Following recovery (16 h) cells ($5\times10^6$ per sample) were stimulated with IL-1 (10 ng/ml for 6 h) or left untreated at which point RNA extractions were performed. (A,B) Duplicate samples were treated with actinomycin D (1 μM) for a further 6 h. The levels of IL-2 mRNA were estimated by RT-PCR using IL-2-specific primers in reactions containing 1 μg total RNA as template. Control reactions measured levels of aldolase mRNA. (A) Products were electrophoresed in 1.5% TAE agarose gels containing 0.5 μg/ml ethidium bromide, visualised under UV and quantified by densitometry. (B) The RT-PCR products were capillary blotted onto nylon membranes and probed with α-$^{32}$P-labelled IL-2 and aldolase cDNAs. The signals were quantified using image analysis (InstantImager, Packard Instrument) and normalised for the amount of aldolase mRNA present. Results from three experiments (mean ± S.D.) are shown in the lower panel of B. (C) Additional samples were treated with actinomycin D (1 μM) for 3, 6 or 24 h prior to RNA extraction. 1 μg amounts of RNA were reverse transcribed into cDNA and used as templates in PCR reactions containing a known quantity of exogenously synthesised competitive IL-2 cDNA (ICS), using biotinylated IL-2-specific primers. The levels of IL-2 and ICS cDNA were quantified by ELISA using capture IL-2- or ICS-specific oligonucleotides bound to microtitre wells. The PCR products were quantified spectrophotometrically at 450 nm following addition of streptavidin-HRP and substrate. The number of copies of IL-2 mRNA were calculated from the ratio of total IL-2 optical density:total ICS optical density and the input number of ICS (2000 copies). Results are expressed as the mean ± S.D. ($n=3$) of relative IL-2 mRNA amounts. At 6 h IL-1 vs. IRAK and TRAF-6, $P=0.0003$ and 0.0001 (**), respectively ($t$-test). At 24 h IL-1 vs. IRAK or TRAF-6, $P<0.0001$ (***).

### 3.5. Effects of IL-1, IRAK and TRAF-6 on IL-2 mRNA production and stability

Considering that IRAK and TRAF-6 expression increased IL-2 promoter-linked reporter gene expression but failed to increase IL-2 protein levels we next investigated their effects on IL-2 mRNA expression. RT-PCR using IL-2 specific primers was performed on RNA samples isolated from control cells and cells transfected with IRAK or TRAF-6 cDNAs (a representative result is shown in Fig. 4A). The relative amount of IL-2 transcript in each sample was determined semi-quantitatively by densitometry. The amounts of IL-2 and aldolase RNA were also quantitated by instant imaging following Southern blotting of the RT-PCR gels onto nylon membrane and probing with $\alpha$-$^{32}$P-labelled IL-2- and aldolase-specific probes (a representative result is shown in Fig. 4B). The steady state level of IL-2 mRNA was low in unstimulated EL4.NOB-1 cells (Fig. 4A,B, lane 1). Expression of IRAK and TRAF-6 induced IL-2 gene transcription $2.5 \pm 0.1$ and $1.6 \pm 0.3$-fold above control (mean $\pm$ S.D., $n = 3$), respectively (lanes 5 and 7). IL-1 treatment (10 ng/ml for 6 h) increased IL-2 mRNA $3.6 \pm 0.3$-fold over control (lane 3). These semi-quantitative measurements were carried out on the Southern blots with similar results being obtained from the quantitation of the RT-PCR products. The level of IL-2 mRNA was normalised relative to the aldolase mRNA in each sample. IRAK and TRAF-6 expression had no effect on transcription of the control aldolase transcript (Fig. 4A,B). These results indicated that both IRAK and TRAF-6 increase IL-2 gene transcription.

We also compared the stability of the mRNAs in the different samples above to determine whether there was a difference in how they were regulated post-transcriptionally. Control, IL-1-stimulated and IRAK- or TRAF-6-transfected cells ($5 \times 10^6$/ml) were treated with actinomycin D (1 mM) to inhibit new transcription. Total RNA was isolated 6 h post treatment and RT-PCRs (Fig. 4A, lanes 2, 4, 6, 8) followed by Southern blotting (Fig. 4B) were carried out. The stability of the IL-2 mRNAs in each sample was measured by calculating its decay after 6 h compared to its accumulation prior to addition of the transcription inhibitor. All values were normalised to the aldolase signals. As already described the steady state level of IL-2 mRNA was low in unstimulated EL4.NOB-1 cells (lane 1); however, these control levels decayed by $50 \pm 7.1\%$ (mean $\pm$ S.D., $n = 3$), following actinomycin D treatment, a representative result being shown in Fig. 4A,B (lanes 1 and 2). The mRNA induced by IL-1 was stabilised, however, with only $18.9 \pm 8\%$ decay (mean $\pm$ S.D., $n = 3$) under the conditions tested (Fig. 4B, lanes 3 and 4). In comparison, the IL-2 transcripts generated as a result of IRAK (lanes 5 and 6) and TRAF-6 (lanes 7 and 8) overexpression were markedly decayed over the same time period. This result meant that the IL-2 mRNA induced by IL-1 was virtually intact at 6 h following actinomycin D, but that induced by IRAK- and TRAF-6-transfected cells had degraded to less of the IL-2 mRNA in unstimulated non-transfected cells.

In order to more rigorously measure the kinetics of IL-2 mRNA decay in response to IL-1 treatment and IRAK or TRAF-6 transfection, time course studies using competitive PCR followed by quantitative microtitre plate measurement of IL-2 and control amplicons were carried out. IL-1-stimulated and IRAK- or TRAF-6-transfected cells were treated with actinomycin D, and total RNA was isolated at various times up to 24 h. cDNA was synthesised using an oligo($dT_{15}$) primer and its integrity was verified by measuring the relative amounts of aldolase mRNA in each sample by PCR using aldolase-specific primers (not shown). Competitive PCR reactions using biotinylated IL-2-specific primers were carried out using a known quantity of exogenously synthesised IL-2 DNA, the ICS, mixed with the sample cDNA. Following PCR the amplicons were visualised by agarose gel electrophoresis (not shown). The PCR products were denatured and hybridised to either ICS or IL-2 sequence-specific capture oligonucleotides bound to microtitre wells and were detected and quantified spectrophotometrically following addition of an enzyme-streptavidin conjugate and substrate. The signal generated in each reaction was proportional to the amount of amplicon present. The ICS served as a standard for IL-2 cDNA quantitation as it was amplified at an efficiency identical to the IL-2 cDNA. The number of copies of IL-2 in each PCR reaction was calculated from the ratio of the total optical density for the IL-2-specific wells to the total optical density for the ICS-specific wells and the in-

put copy number of the ICS. These results were expressed as relative IL-2 mRNA amounts. As shown in Fig. 4C the relative amount of IL-2 mRNA generated following IL-1 treatment remaining after actinomycin D treatment was $94 \pm 3$, $72 \pm 2.5$ and $59 \pm 0.2\%$ at 3, 6 and 24 h, respectively (mean ± S.D., $n = 3$). In contrast the IL-2 mRNAs generated in response to IRAK or TRAF-6 transfection were considerably less stable. The IL-2 mRNA generated following IRAK transfection decayed by $66 \pm 1.7\%$ at 3 h, and less than 1% remained after 24 h. TRAF-6-induced IL-2 mRNA also decayed more rapidly then the IL-1-induced IL-2 mRNA, with less than 8% remaining 6 h post actinomycin D treatment. These results confirm that IL-2 mRNA induced by IL-1 is stabilised whilst that induced in IRAK- and TRAF-6-transfected cells is unstable. IL-1 treatment, but not IRAK or TRAF-6, can provide signal(s) that stabilise IL-2 mRNA. Such signals must therefore be essential for the efficient translation of the IL-2 mRNA induced.

## 4. Discussion

Significant advances have been made recently in elucidating components of the IL-1 signalling pathway leading to NFκB activation, providing us with an increased understanding of the molecular processes involved. IRAK, IRAK-2, TAB1/TAK1, NIK and the IKKs represent an array of novel protein kinases which signal in cooperation with novel signal transducing molecules, TRAF-6 and MyD88, to coordinate IL-1-stimulated intracellular responses [5,8,12,13,15–23]. The involvement of these factors has added an increased complexity to the emerging picture of how IL-1 induces its pleiotropic biological effects. The expression of over 90 genes affecting immune and inflammatory responses has been shown to be affected by IL-1 and an area of intense study has been the receptor-mediated signal transduction pathways inducing such changes in gene expression. Analysis of the cellular regulation of the newly characterised factors will help to establish an even clearer picture of how IL-1 signalling occurs. Experiments described here were designed to investigate the involvement of two IL-1 signal mediators, IRAK and TRAF-6, in IL-1-induced responses in EL4.NOB-1 T cells, namely induction of the IL-2 promoter, IL-2 mRNA and protein. We have found that IRAK and TRAF-6 regulate the IL-2 gene, their primary effect being at the level of transcription. We provide direct evidence that TRAF-6 lies downstream from IRAK, and that TRAF-6 is critical for IRAK- and IL-1-induced responses. We show that IL-1 controls post-transcriptional regulation of IL-2 by providing stabilisation signals for IL-2 mRNA which are required for IL-2 protein production. Such signals are unlikely to involve IRAK or TRAF-6.

Most IL-1-responsive genes carry consensus sequences for NFκB in their promoters making it an obvious target molecule to monitor when examining IL-1-induced effects. The elucidation of NIK as a regulator of IKK1 and 2, the enzymes responsible for phosphorylation of IκBα [21,22], has clearly defined the molecular events which occur directly upstream from NFκB, with IRAK and TRAF-6 appearing to act in proximity to the membrane-associated IL-1 receptor complex. The ability of IRAK to induce NFκB-linked reporter gene expression in EL4.NOB-1 cells was not surprising considering the similar reports of both IRAK's and IRAK-2's effect on NFκB in other cell lines [5,13]. However, the potency of its effect on NFκB in the EL4.NOB-1 cells is of interest. The powerful activation of a downstream target by IRAK even when expressed at low levels indicates that it commands an apical position in the pathway. The TRAF-6 effect on NFκB-mediated transcription was somewhat less pronounced than that of IRAK, with higher plasmid concentrations being needed to induce an optimal response.

The IRAK transfection experiments also indicated that large amounts of IRAK were toxic to the cells. This was not seen when equivalent doses of TRAF-6 or vector were substituted, indicating that IRAK can induce cell killing at high doses. IRAK contains a death domain which could be responsible for generating death signals through associations with the cell's apoptotic machinery. Indeed, IL-1 has been shown to induce cytotoxic effects in certain cell types, e.g. insulin-producing islet β cells [28]. This may be accomplished by transducing a death signal through IRAK.

Another surprising observation in IRAK-transfected cells was the IL-1-independent phosphoryla-

tion of IRAK seen following transfection, indicating a sensitive sensing mechanism controlling activation of IRAK in EL4.NOB-1 cells. Translocation of IRAK to the IL-1 receptor complex occurs prior to or independently of IRAK phosphorylation. As it becomes associated with the receptor complex IRAK becomes increasingly phosphorylated and simultaneously activated [29]. We speculate that overexpression of IRAK from a constitutive CMV promoter is responsible for the observed effect. Relatively small amounts of transfected IRAK cDNA induced activation hinting at the existence of a sensitive detector responsible for regulating IRAK phosphorylation. Alternatively, IRAK overexpression may induce spontaneous IL-1RI and IL-1RAcp receptor aggregation thereby mimicking IL-1 engagement of its receptors.

The ability of IRAK and TRAF-6 to cause activation of NFκB in EL4.NOB-1 cells prompted further analysis into what additional transcriptional effects IRAK or TRAF-6 could induce. Expression of either protein had no effect on an NFAT-linked reporter gene in the absence or presence of an IL-1 stimulus (data not shown). Expression of IRAK or TRAF-6 induced the IL-2 promoter. This induction was inhibited by cotransfection with a S32A/S36A mutant of IκB which prevents NFκB activation and showed that the IL-2 promoter is critically dependent on NFκB. IRAK and TRAF-6 expression also increased IL-2 mRNA production. However, interestingly, neither IRAK nor TRAF-6 increased IL-2 protein production. Nonetheless, this IL-1 response was potentiated in cells overexpressing IRAK and TRAF-6. A recent study in EL4 6.1 cells stably transfected with IRAK gave a similar result with regard to IL-2 protein production where the IL-1 response was potentiated [30]. This indicated that both IRAK and TRAF-6 were participating in the pathway to IL-2 production but that their effects were primarily transcriptional.

Additional signals were shown to be induced by IL-1 which have post-transcriptional effects on the IL-2 mRNA. These signals include stabilisation of the IL-2 mRNA, an event which was not induced by expression of IRAK or TRAF-6. Our initial investigations suggested that IL-1 treatment led to stabilisation of the IL-2 transcript but that IRAK or TRAF-6 expression failed to stabilise the IL-2 mRNA under the same conditions. Quantitative competitive PCR confirmed this observation and demonstrated that IL-2 mRNAs generated following IRAK or TRAF-6 transfection were far less stable than those induced by IL-1. This implied that neither IRAK nor TRAF-6 can regulate processes involved in IL-2 mRNA stabilisation. Such processes may involve MAP kinases such as JNK or p38 MAP kinase, and IRAK has been shown by others to regulate both kinases [30–33]. JNK has been implicated in stabilisation of IL-2 mRNA. However, this was not examined in response to IL-1 stimulation [34]. Our result implies that neither kinase is involved here or alternatively that additional signals are needed which are not activated by IRAK or TRAF-6.

Our previous report that IRAK and TRAF-6 induce AP1-linked gene expression and potentiate the effect of IL-1 on this response suggests that IRAK and TRAF-6 can integrate additional signals from separate pathways [14]. These effects on AP1 may involve JNK. The TRAF-6-mediated activation of both NFκB and AP1 in response to IL-1 is similar to TNF-induced effects on the TRAF2 signal transducer. TRAF2 represents the bifurcation point of independent pathways which culminate in NFκB and AP1 activation [35]. It is possible that TRAF-6 fulfils a similar role in transduction of the IL-1 signal to distinct downstream effectors. Alternatively, TRAF-6 might mediate AP1 activation via NIK in a JNK-independent manner similar to the TNFR1-mediated TRAF2 pathway [35]. In B cells CD40-mediated NFκB activation can occur by two different mechanisms. One pathway involves TRAFs 2, 3 or 5 and requires the TIMct region of CD40 [10,36–38] whereas the other is a TRAF-6-dependent mechanism and involves TRAF-6 recruitment to the amino terminal end of CD40 [27]. Whether IRAK is involved in this pathway is not known. Nonetheless it appears that TRAF-6 may have a more general function as an adaptor protein which participates in different signalling pathways.

All of the responses tested in this study were TRAF-6-dependent as shown by their inhibition following expression of TRAF-6Δ. Overexpression studies have demonstrated the ability of TRAF-6Δ to inhibit IRAK-2 induced NFκB activation [13]; however, no IL-1-dependent effects were tested. Here, similar to the report of TRAF-6 functioning

downstream from Myd88 [39], we provide direct evidence that TRAF-6 lies downstream from IRAK. Previous evidence in this regard has been circumstantial, as it has involved immunoprecipitations [8]. Unlike IRAK, TRAF-6 does not co-immunoprecipitate with IL-1RI. IRAK and TRAF-6 will co-immunoprecipitate, however. Our results confirm the proposed model that TRAF-6 lies downstream from IRAK. Our results also show that TRAF-6 mediates activation of the IL-2 promoter by both IRAK and IL-1. TRAF-6Δ did not completely abolish the effects of IRAK or IL-1. It is likely therefore that other signals participate in the responses mediated. These can function when downstream targets of TRAF-6 are not activated. The inhibitory effect of TRAF-6Δ on the IL-1-induced increase in IL-2 protein production clearly pointed to the importance of TRAF-6 for IL-2 gene transcription activated by IL-1.

As yet, apart from IL-1 the only other stimulus identified which activates IRAK is IL-18 [40,41]. IL-18 signals NFκB activation through a receptor that associates with IRAK in Th1 cells but which is distinct from IL-1RI. This receptor has been suggested to be IL-1Rrp, a homologue of IL-1RI [42]. Indeed, in EL-4 cells binding of IL-18 to this receptor activates the IRAK-TRAF-6 pathway resulting in NFκB activation [43]. Medzhitov et al. described upregulation of NFκB-controlled genes by constitutively active hToll (hTLR4) [44], also a member of the IL-1 receptor family. hToll has been implicated in the integration of innate immunity and induction of adaptive responses [45] with IRAK and TRAF-6 participating in this response [46]. More recently human TLR2 and murine TLR4 have been shown to mediate LPS signalling [47,48]. Therefore IRAK and TRAF-6 are likely to be crucial signalling molecules for other members of the IL-1 receptor family, including the growing subset of human Toll-like receptors (hTLRs) [49].

In conclusion, this study points to a critical role for IRAK and TRAF-6 in regulation of IL-2 gene expression. Their effects are on transcription with additional IL-1 signals being responsible for post-transcriptional events, including mRNA stabilisation, leading to IL-2 protein production. Both IRAK and TRAF-6 can therefore be added to the list of signal transducers essential for T cell activation and are likely to be important regulators of inflammation and innate immunity.

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