Simultaneous Blockade of NFκB, JNK, and p38 MAPK by a Kinase-inactive Mutant of the Protein Kinase TAK1 Sensitizes Cells to Apoptosis and Affects a Distinct Spectrum of Tumor Necrosis Target Genes*‡

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The inflammatory response is characterized by the induction (or repression) of hundreds of genes. The activity of many of these genes is controlled by MAPKs and the IκB kinase-NFκB pathway. To reveal the effects of blocking these pathways simultaneously, fibroblasts were infected with retroviruses encoding TAK1K63W, an inactive mutant of the protein kinase TAK1. Expression of this protein inhibited tumor necrosis factor (TNF)-induced activation of NFκB, JNK, and p38 MAPK and sensitized the cells to TNF-induced apoptosis. 23 different microarray experiments were used to analyze the expression of >7000 genes in these cells. We identified 518 genes that were regulated by TNF in both TAK1K63W-expressing cells and control cells, 37 genes induced by TNF only when TAK1K63W was present, and 48 TNF-induced genes that were suppressed by TAK1K63W. The TNF-inducible genes that were most strongly suppressed by TAK1K63W, ccl2, ccl7, ccl15, cxcl11, cxcl5, cxcl10, saa3, and slpi also had much lower basal levels of expression, indicating that TAK1 also played a role in their normal expression. Chromatin immunoprecipitation studies on four of these genes suggested that inactivation of TAK1 activity led to direct suppression of expression at the transcriptional level because of impaired recruitment of RNA polymerase II to their promoters. ccl2 induction by TNF or interleukin-1 was also suppressed in cells that expressed TAK1 antisense RNA or that were genetically deficient in JNK1/2 or p65 NFκB. These data suggest that regulation of the expression of a selected group of inflammation-related genes is funneled through TAK1, making it a potentially useful target for more specific anti-inflammatory drug development.

Inflammation is a component of the innate immune system that produces a local protective response to microbial invasion or injury. One hallmark of inflammation is a profound change in the expression of a large number of secondary inflammatory proteins such as chemokines, cytokines, proteases, and metabolic enzymes (e.g. cyclooxygenases 1 and 2 and nitric-oxide synthase) (1). These genes are induced by molecular pattern recognition receptors such as Toll-like receptors (TLR) that detect viral, bacterial, or fungal infections or by proinflammatory cytokines such as IL-1 and TNF (2, 3).

The signaling receptors for IL-1 and TLR have a conserved cytoplasmic domain called the Toll/IL-1R (TIR) domain and, therefore, form the TIR superfamily (3–5). The principal activation mechanism of these receptors is ligand-induced oligomerization, resulting in the homophilic interaction with cytoplasmic adapters such as MYD88 and TNF receptor-associated factor (TRAF) 6. TIR receptors then couple to and activate IL-1-receptor-associated protein kinases 1–4 (3–6). Similarly, TNF receptors trimmerize and then interact with the adapters TRADD and TRAF2 followed by recruitment of the protein kinase RIP (7, 8).

All of the TIR receptors and TNF receptors activate the IκB kinase (IKK)-NFκB, JNK, p38, and ERK MAPK signaling pathways. These kinases regulate the nuclear activation of subunits of the NFκB and AP-1 families of transcription factors (1). NFκB- and AP-1-binding sites have been found in the promoters of many genes that are induced during inflammation, which has led to the view that a large number of specific receptors of the innate immune system exert their manifold gene activations principally through the MAPK and the IKK-NFκB pathways (1, 9).

As expected from this conclusion, IL-1, TNF, and TLRs induce common sets of genes such as cyclooxygenases, chemo- kines, and other cytokines (2). However, these three mediators...
of inflammation induce quite distinct biological responses, e.g., apoptosis is induced by TNF but not by IL-1, and many genes are uniquely induced in response to TLRs (10). One important and unresolved question concerning inflammatory signal transduction is into which gene regulatory response pattern(s) activation of NFκB and MAPK pathways by the TIR and TNF receptors is actually translated.

Three types of molecules specifically couple these receptors to their downstream signaling pathways: 1) adaptors such as TICAM, TIRAP/MAL, and TRIF (11, 12); 2) the four IL-1 receptor-associated protein kinases (IRAK) (6), and 3) the large number of MAPK kinase kinases (MAPKKK) (13).

The MAPKKK TAK1 was originally found to be activated by transforming growth factor-β (14); however, there is increasing evidence that it is also activated by a variety of proinflammatory stimuli such as IL-1 (15), IL-18 (16), lipopolysaccharide (17), poly(I-C) (18), bacteria (19), and RANKL (20) among others. The molecular mechanisms by which TAK1 is coupled to the IL-1 receptor have been analyzed in detail. TAK1 is constitutively associated with at least three TAK1-binding proteins (TAB1–3). Upon activation of the IL-1 receptor complex, the TAK1-TAB complex binds to TRAF6 and TAK1 becomes activated, a process that requires ubiquitination of TRAF6 and the ubiquitin ligase activity of TRAF6 (21–25). Rather than targeting these molecules to proteasomes for destruction, ubiquitination stabilizes the TRAF6-TAK1-TAB (1–3) interactions and activates autophosphorylation of TAK1 (26). This phosphorylation then activates TAK1, which in turn activates the IKK-NFκB, JNK, and p38 MAPK pathways via key phosphorylations. Considerably less information exists on the mechanism of TAK1 activation by the TNF receptor, although suppression of TAK1 by RNAi recently suggested that this kinase is also important for TNF-mediated NFκB activation (27, 28).

In contrast, despite the large body of biochemical evidence implicating TAK1 in signaling of proinflammatory stimuli, virtually no information exists on the specific aspects of inflammation that require TAK1 activity.

We have recently shown using a dominant negative TAK1 mutant, TAK1K63W, that TAK1 controls the transcription and mRNA stabilization of the human chemokine interleukin-8. To extend these results, we decided to investigate the impact of this mutant on inflammatory gene responses utilizing a large number of comparative microarray experiments.

Here we report that TAK1 controls the basal and inducible expression levels of a specific set of inflammatory genes. We further provide evidence that TAK1K63W affects chemokine gene expression by a complex transcriptional mechanism that involves mainly the NFκB and JNK pathways. These results contribute substantially to our understanding of the molecular mechanisms by which MAPKKK selectively couple receptors of the nonspecific immune system to gene regulatory responses. They also suggest approaches that may have the potential for designing targets for therapeutic intervention in inflammation.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials—**NIH3T3 cells were obtained from DSMZ (German collection of microorganisms and cell culture) (DSMZ no. ACC 59). GP + E86 cells were obtained from A. Berns. NIH3T3/TA cells stably expressing the tet transactivator protein were a kind gift of H. Bujard, respectively. Fibroblasts deficient for JNK1/2, p38 MAPKα, and p65 NFκB were kind gifts of Erwin Wagner (Vienna, Austria), Angela Nebreda (Heidelberg, Germany), and Hiroyasu Nakanoto (Tokyo, Japan), respectively. All of the cells were cultured in Dulbecco’s modified Eagle’s medium complemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Antibodies against the following proteins were used in this study: polyethylene II (sc-899, Santa Cruz Biotechnology); GFP (clones 7.1 and 13.1, Roche Applied Science); AKT, and phospho-AKT (Pharmingen).

Human recombinant IL-1α and rabbit antiserum was made to synthetic peptides for the C terminus of JNK2 (peptide DSSDLASTPGLL, amino acids 409–423) or p38 MAPK (peptide ISFPPLDQLQEMEES, amino acids 346–360) were kind gifts of J. Saklatvala (Cambridge, United Kingdom). The expression plasmid for GST-JUN (amino acids 1–135) was a kind gift of J. R. Woodgett (Ontario, Canada). GST-JUN was expressed and purified from Escherichia coli by standard methods.

Recombinant bacterially expressed His epitope-tagged MAPK-activated protein kinase-2 was a kind gift of M. Gaestel (Hannover, Germany). Horseradish peroxidase-coupled secondary antibodies and protein A/G- Sepharose (from Amersham Biosciences) were from Amersham Biosciences. TNF-α was from a gift from W. D. Stadler (Wuerzburg, Germany). FASL-containing medium was a kind gift of David Wallach (Rehovot, Israel) and was used in a 1:2 dilution for all of the experiments. Z-VAD-fmk was from Calbiochem, and wortmannin was from Alexis. The cDNA Northern probes for ccl2 (268 bp), ccl7 (365 bp), and ccl3 (320 bp), slpi (366 bp), and gapdh (1400 bp) were produced by reverse transcription-PCR. Other reagents were from Sigma or Fisher and were of analytical grade or better.

**Plasmids and Transfections—**The cDNAs of GFP-TAK1 or GFP-TAK1K63W were amplified from pEGFP-TAK1 expression plasmids (29) by PCR using the primers (sense 5′-GGGATCCTAGGATCCATTCTTCTTTGTCAAGGAGG-3′; antisense 5′-GGGGATCCGTGATTTCTCCACATTCTTCACTTCTGCCTGTTCT-3′) and cloned into the C1a site of pM5Xneo, a myeloproliferative sarcoma virus-based retroviral vector that contains a poliovirus internal ribosomal entry site/neomycin phosphotransferase cassette for the convenient selection of cells.2 The plasmids were stably transfected into GP + E86-packing lines, and virus-containing supernatants were used to infect NIH3T3 cells in the presence of 8 μg/ml Polybrene. Additionally, GP + E86 cells were transfected with pM5Xneo without TAK1 cDNAs and retroviruses derived thereof were used to stably infect "empty virus" control cells. Stably infected cells were selected in 1 mg/ml G418. The GFP-TAK1 cDNA fragment was also cloned in an antisense orientation into the C1a site of pH1-L (Clontech). Sall was used to reduce a 775-bp fragment containing the GFP cDNA to generate pBI-LTAK1a. In this vector, a tetracycline-responsive bidirectional promoter controls the expression of luciferase and TAK1a cDNA. Stably transfected cell lines were generated by cotransfection of pBI-LTAK1a and pBSAPC, a derivative of PSV2P4, into NIH3T3/TA. Cellular clones were obtained by limiting dilution in selection medium (0.75 μg/ml puromycin, 2 mg/ml tetracycline) and identified by luciferase expression. Transient transfections by the calcium phosphate method and determination of luciferase reporter gene activity were essentially performed as described (29). For determination of NFκB-driven promoter activity, cells (seeded at 5 × 10<sup>4</sup>/well of 6-well plates) were transfected with 0.25 μg of pNFκBp65lcu or with 0.5 μg of pGAL4(2)/Luc and 0.5 μg of pGAL4p65 that encodes for p65 fused to the DNA-binding domain of GAL4. These vectors were a kind gift of Lienhard Schmitz (Bern, Switzerland). Empty pCS3MT vector was added to bring the total DNA per well to 5 μg. For normalization of transfections, 0.5 μg of pSV-β-galactosidase (Promega) was cotransfected and β-galactosidase activity was measured using a kit from Clontech. For the experiments shown in Fig. 7, equal molar amounts of luciferase reporter plasmids according to Wagner et al. (31) were transfectected together with 2.5 μg of pSV-β-galactosidase. Equal amounts of plasmid DNA within each experiment were obtained by adding empty vector.

**Chromatin Immunoprecipitation (ChIP)—**Three 175-cm<sup>2</sup> flasks of confluent NIH3T3 cells, treated as described in the figure legends, were used for each condition. Proteins bound to DNA were cross-linked in vivo by replacement of the medium with warm phosphate-buffered saline containing 1% formaldehyde. After incubation for 5 min at room temperature, this solution was replaced by cold phosphate-buffered saline containing 0.125% glycine to stop the cross-linking. The supernatant was removed and the cells were washed in and scraped into ice-cold phosphate-buffered saline. Cells were collected at 500 × g at 4 °C, washed again in ice-cold phosphate-buffered saline, and then lysed in ChIP-radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, and freshly added 1% proteinase). Lysates were cleared by sonication (4 × 1 min on ice) and centrifuged at 15,000 × g at 4 °C for 20 min. Supernatants were collected and stored in aliquots at −80 °C for subsequent ChIP. 10 μl of antibodies were added to 500 μl of lysates, and the mixture was rotated at 4 °C overnight. 40 μl of a protein A/G mixture preequilibrated in ChIP-radioimmunoprecipitation assay buffer then was added to the lysates, and incubation continued for 2 h.

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2 D. Wirth, unpublished data.
at 4 °C. Beads were collected by centrifugation, washed twice in 1.4 ml of ChIP-radioimmunoprecipitation assay buffer, once in high salt buffer (10 mM Tris, pH 7.5, 2 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 1 mM EDTA), once in ChIP-radioimmunoprecipitation assay buffer (cold), and once in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at room temperature, and finally resuspended in 55 μl of elution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS). Samples were vigorously mixed for 15 min at 25 °C and then centrifuged. 50 μl of supernatant was diluted to 200 μl with TE buffer including RNase A (50 μg/ml). Similarly, 50 μl of the initial lysate (input samples) were diluted to 200 μl with TE buffer containing 1% SDS and 50 μg/ml RNase A. After 30 min at 37 °C, proteinase K was added (0.5 mg/ml) and both input and immunoprecipitated were incubated for at least 6 h at 37 °C followed by at least 6 h at 65 °C. Samples were diluted to 500 μl in loading buffer (Qiagen), and DNA was purified using QIAQuick spin columns (Qiagen) according to the manufacturer’s instructions. DNA was eluted with TE buffer and stored at −20 °C until further use. PCR was performed on input and immunoprecipitated DNA using 2.5 μl of hostTaq polymerase (Qiagen), 0.5 μM sense and antisense primer, 2.5 μl of template DNA, and 0.2 mM dNTPs in a total volume of 30 μl. PCR cycles were as follows: 15 min at 95 °C and 30–35 cycles of 94 °C (20 s), 55–60 °C (20 s), and 72 °C (20 s) followed by a final extension reaction at 72 °C for 7 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining, and fluorescence intensities of bands were quantified using a Bio-Imager system (Biomat) and the BioDocAnalyze software, version 1.0 (Biomat). cDNA was isolated from each sample using a High Pure RNA isolation kit (Roche Applied Science) followed by “on-column” DNase I digestion. RNA was used to prepare Cy3-labeled cRNA by oligo(dT)-T7-primed double-stranded cDNA synthesis (cDNA synthesis system, Roche Applied Science) followed by in vitro transcription with T7 polymerase (MEGAScript T7 kit, Ambion) as directed by the manufacturer. The cRNA yield was determined photometrically. Fluorescence intensity values for cellular genes that exceeded this threshold by two standard deviations (corresponding to values of at least 104 arbitrary fluorescence units) were regarded as significant expression signals.

**RESULTS**

**Stable Expression of GFP-TAK1K63W Inhibits NFκB, JNK, and p38 MAPK Activation and Induces Apoptosis in Response to TNF**—To investigate potential gene-regulatory functions of TAK1, we constructed retroviral expression vectors of a previously described fusion protein of GFP with a catalytically inactive mutant of TAK1, TAK1K63W (29). NIH3T3 fibroblasts were stably infected with empty retrovirus or with TAK1K63W-containing retroviruses yielding pools of cell lines that strongly overexpressed the inactive GFP-TAK1 mutant (Fig. 1A). Initially, we analyzed the major known IL-1- and TNF-induced signal transduction pathways in these cells. As expected, the expression of GFP-TAK1K63W inhibited IL-1- and TNF-induced activation of IKK and nuclear translocation of NFκB as assessed by electrophoretic mobility shift assay (supplemental Fig. 1A). It also strongly suppressed the activation of a transiently transfected luciferase reporter gene driven by three NFκB sites in response to IL-1 or TNF (Fig. 1B). Of note, TAK1K63W also suppressed the basal activity of this

**Immunoprecipitation—**Immunoprecipitation was performed using FITC-conjugated rabbit antibodies against JNK or p38 MAPK followed by the addition of 20 μl of a 50% suspension of protein A-Sepharose beads and incubation for 1–2 h at 4 °C. Beads were spun down, washed three times in 1 ml of immunoprecipitation buffer A, and resuspended in 10 μl of the same buffer. 1 μg of recombinant protein substrates (GST-JUN D135 or HISSMPK-activated protein kinase)–2 in 10 μl of H2O and 10 μl of kinase buffer (150 mM Tris, pH 7.4, 0.1 mM Na3VO4, 0.4 mM Na2P2O7, 0.5 mM MgCl2, 0.2 mM MnCl2, 60 μM ATP, and 10 μl of [γ-32P]ATP) were added. After 30 min at room temperature, SDS-PAGE sample buffer was added and proteins were eluted from the beads by boiling for 5 min. After centrifugation at 10,000 × g for 5 min, supernatants were separated on 10 or 12.5% SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

**Stick Blotting—**Stick blotting was performed exactly as described previously (34). A negative control threshold was calculated from the mean fluorescence intensities obtained in control Arabidopsis oligonucleotides. Fluorescence intensity values for cellular genes that exceeded this threshold by two standard deviations (corresponding to values of at least 104 arbitrary fluorescence units) were regarded as significant expression signals.
construct (Fig. 1B) as well as that of a transiently transfected GAL4p65 fusion protein (Fig. 1C). TAK1K63W also inhibited the phosphorylation of endogenous p65 NF-κB at Ser-536 (supplemental Fig. 1B), which contributes to NFκB-mediated transactivation (27, 35). These data indicate that TAK1 controls basal as well as inducible NFκB activity by affecting several levels of the NFκB signaling pathway. The activation of JNK in response to TNF or IL-1, as assessed by immune complex protein kinase assays, was completely blocked by expressing the catalytically inactive TAK1 mutant (Fig. 1D). In addition, we found that the modest TNF- or IL-1-stimulated activation of p38 MAPK in these NIH3T3 lines was also significantly suppressed by the TAK1 mutant (Fig. 1E). Interestingly, the phosphorylation of the protein kinase AKT at Ser-473, which is a downstream effect of the activation of PI3K, was stimulated by both IL-1 and TNF in the presence or absence of TAK1K63W expression, indicating that (i) the inhibitory effect of dominant negative TAK1 was specific for NFκB, JNK, and p38 MAPK and that (ii) there are TAK1-independent IL-1/TNF-responsive pathways (Fig. 1F). Retroviral overexpression of wild-type GFP-TAK1 at levels comparable to those achieved with GFP-TAK1K63W did not interfere with NFκB activation (supplemental Fig. 1A) or AKT phosphorylation (Fig. 1F).

In culture, the cells expressing the dominant negative TAK1 did not show any obvious abnormalities in shape, adherence, or proliferation (data not shown), suggesting that the TAK1K63W mutant did not affect any serum-mediated cellular responses required for survival. However, as expected from the essential role of NFκB in TNF-mediated regulation of survival genes, cells expressing TAK1K63W were highly sensitized to TNF-induced apoptosis. Cell death occurred after ~4 h of treatment with 20 ng/ml TNF (Fig. 2A), and significant evidence of cell death could be detected after overnight treatment with TNF at doses as low as 20 pg/ml (Fig. 2B). Apoptosis was as strong as that induced by simultaneous treatment of cells with FASL and cycloheximide (Fig. 2C). In contrast, cells transfected with empty virus did not die in response to TNF (Fig. 2). Both TNF and FASL-induced apoptosis occurred by caspase-dependent mechanisms as shown by its inhibition by the broad-spectrum caspase inhibitor Z-VAD-fmk (Fig. 2C). Thus, the extensive biological consequences of expression of TAK1K63W, i.e. simultaneous blockade of three major signaling pathways (Fig. 1) and sensitization to apoptosis (Fig. 2), became apparent only if cells were exposed to TNF, suggesting that TAK1 may have stimulus-specific functions.

**TAK1K63W Affects a Specific Set of TNF-induced Genes**—Based on these results, we anticipated that a large number of TNF-regulated genes should be affected by the TAK1K63W mutant. To identify these genes systematically, we performed a series of replicate high-density cDNA microarray experiments.

**Identification of TAK1 Target Genes**—To identify these genes systematically, we performed a series of replicate high-density cDNA microarray experiments.
Cells stably transfected with empty virus or cells expressing TAK1K63W were left untreated or stimulated with TNF for 4 h. cDNAs from stimulated and unstimulated cells were prepared from total RNA, labeled, and cohybridized onto the same microarray. For each of these hybridizations, a duplicate experiment was performed on RNA isolated from a completely independent set of cells/treatments. The experiments of control cells or of the TAK1K63W-expressing cells were then assigned to two groups. By the spot-filtering criteria applied, 7569 or 7417 genes were suitable for further analysis from TAK1K63W-expressing cells or control cells, respectively. Of these, 7260 were analyzable in both groups (Fig. 3A). A groupwise comparison revealed that 1585 genes in control cells and 842 genes in TAK1K63W-expressing cells were differentially expressed by at least 2-fold after TNF treatment, indicating that TAK1K63W affects a large number of TNF-regulated genes (Fig. 3B).

As revealed by scatter plot analysis, these TNF-mediated changes occurred reproducibly in both of the two independent microarray experiments with correlation coefficients of $r = 0.956$ (for ratios of control cells) and $r = 0.972$ (for ratios of TAK1K63W-expressing cells) (supplemental Fig. 3). A Boolean comparison revealed that TNF treatment altered the expression of 518 genes by >2-fold in both cell lines (Fig. 3B). These genes fell into one cluster of 264 genes that were down-regulated and another cluster of 254 genes that were up-regulated (Fig. 3C and supplemental Table I). The analysis of this group of 518 genes revealed that the ratio of altered gene expression was very similar among the four individual experiments. Examples of this finding can be seen in Fig. 3C where the original spot images are shown along with the corresponding ratios of relative gene expression for 20 individual genes selected from the cluster analysis.

This result was intriguing, because it suggested that despite the simultaneous inhibition of NFκB, JNK, and p38 in TAK1K63W cells, the TNF-mediated regulation of a substantial set of genes was unaffected by inhibition of these major signaling pathways. However, in these cDNA microarray experiments, gene regulation is defined as the ratio of gene expression between basal (unstimulated) and TNF-stimulated levels. This finding does not rule out the theoretical possibility that the genes that appear normally regulated by TNF in TAK1K63W-expressing cells actually exhibited both an altered basal as well as an altered stimulated expression level. This situation could mask the identification of some genes that actually are regulated in a TAK1-dependent manner. To test this possibility, we repeated the microarray experiments using a different design, i.e. by hybridizing cDNA from TNF-stimulated control cells and TNF-stimulated TAK1K63W cells directly onto the same microarray. To further strengthen the
results, a dye-swap experiment was included the experimental design. The resulting ratios were then filtered to analyze only those TNF-responsive genes that were either induced or suppressed at least 2-fold more in the TAK1K63W mutant-expressing cells than in the control cells in all three experiments. Cluster analysis revealed 26 genes that were expressed at least 2-fold lower \((/H11002)\) in the TAK1K63W-expressing cells after TNF treatment and 37 genes that were at least 2-fold higher \((/H11001)\) than TNF-treated control cells. Fig. 4A shows the cluster analysis and the corresponding original spot images. Ratios of fluorescence intensities, gene names, and identifiers are shown in the table. The complete information for the 518 genes is provided in supplemental Table I. \(+\), up-regulated genes; \(-\), down-regulated genes.

**Identification of TAK1 Target Genes**

**Fig. 3.** High density microarray hybridization experiments to identify TAK1-dependent and -independent TNF-induced genes. A, cells infected with empty virus or with virus containing GFP-TAK1K63W were stimulated for 4 h with TNF (20 ng/ml) or left untreated. Total RNA was then extracted and used as template to prepare allyl amino-containing cDNA. cDNA from unstimulated cells was conjugated with Cy3 (green), and that of TNF-stimulated cells was conjugated with Cy5 (red). Green- and red-labeled cDNAs were mixed and hybridized to the NCI cDNA microarray. For each comparison, two experiments were performed with RNAs from independently treated cells. Red and green fluorescence of each spot were measured at 532 and 435 nm, respectively, using an Axon 4000 array reader and normalized to the median intensity of the entire array. A, the total number of analyzed genes from the two experiments from cells expressing empty virus or from the two experiments from cells expressing TAK1K63W, respectively, is shown. 7260 spots were detected in all four experiments. B, the data sets shown in A were filtered to study only genes with expression levels altered at least 2-fold following TNF treatment. By these criteria, 518 genes showed TNF-altered expression in both cells expressing empty virus and cells expressing TAK1K63W. C, these genes were further grouped into 264 TNF-down-regulated (green color) and 254 TNF-up-regulated (red color) genes (left panel) and organized by self-organizing map cluster analysis. Original spot images obtained from the individual microarray experiments (experiments 1 and 2 (Exp. 1 and Exp. 2)) for 20 representative genes are shown in the middle panel. Corresponding ratios of fluorescence intensities, gene names, and identifiers are shown in the table. The complete information for the 518 genes is provided in supplemental Table I. \(+\), up-regulated genes; \(-\), down-regulated genes.
gadd45β, and gadd45γ, were higher in TNF-treated TAK1K63W cells. The genes that were most strongly suppressed by TAK1K63W in TNF-treated cells included ccl7 (MCP-3), saa3, ccl2 (MCP-1), c3, and slpi, suggesting that TAK1 may regulate the expression of a set of genes with known relevance to inflammation.

**Distinct Groups of Inflammatory Genes Are Strongly Suppressed by TAK1K63W and Are Induced by Apoptosis**—Therefore, we aimed to investigate in more depth the role of TAK1K63W in the regulation of inflammatory genes with particular attention to the rapid apoptosis that occurs in conjunction with suppression of NFκB and MAPK signaling. For this, we used a small customized DNA microarray that contains validated oligonucleotide probes for 136 inflammation-related genes. In a total of 16 microarray hybridizations, we analyzed constitutive and TNF-inducible gene expression profiles of control cells (expressing empty virus) and of cells expressing TAK1K63W. In parallel experiments, both cell types were also pretreated with Z-VAD-fmk to assess the contribution of caspase-mediated processes, such as apoptosis, to inflammatory gene expression. Labeled cRNA were hybridized individually to microarrays, allowing the separate analysis of basal

**Fig. 4.** High-density microarray hybridization experiments identify subsets of TNF-induced genes whose expression was up-regulated or down-regulated by TAK1K63W. Cells infected with empty virus or with viruses containing GFP-TAK1K63W were stimulated for 4 h with TNF (20 ng/ml). cDNAs were prepared as described in the legend to Fig. 3 then were labeled with Cy3 (empty virus, green) or with Cy5 (TAK1K63W, red), respectively. Two independent experiments were performed (lanes 1 and 2). In a third experiment, cDNA labeling was reversed (lane 3). Red and green fluorescence of each spot were measured as described in Fig. 3. Cluster analysis was used to organize genes that were differentially expressed by at least 2-fold in all three experiments and whose expression ratio was reversed by the dye-swap experiment. A, cluster analysis and the corresponding original spot images. B, ratios of fluorescence intensities, gene names, and identifiers corresponding to the genes shown in A. Genes are presented in decreasing degree of suppression or induction by TAK1K63W. +, up-regulated genes; −, down-regulated genes. White spot color indicates saturation at that scan intensity.
and TNF-inducible mRNA expression. This analysis revealed that TAK1K63W suppressed basal as well as TNF-inducible expression of 22 TNF-dependent inflammatory genes (Fig. 5). The 10 most strongly suppressed genes included six chemokines (ccl7, ccl2, cxcl5, cxcl1, cxcl5, and cxcl10), indicating that chemokine expression is highly sensitive to TAK1K63W.

In control cells, Z-VAD-fmk had no effect on basal expression levels or the TNF-induced up-regulation of these 22 genes (Fig. 5A). However, in TAK1K63W-expressing cells, the basal levels of expression of nearly all of these genes were significantly lower than in control cells. Nonetheless, TNF induced an up-regulation of most of them. Unexpectedly, Z-VAD-fmk completely inhibited this TNF-induced up-regulation, suggesting that apoptosis partially counteracts the suppressive effect of TAK1K63W on inflammatory genes (Fig. 5B). This clearly indicates that TNF-stimulated increase in ccl7, ccl2, cxcl5, cxcl1, and cxcl10 gene expression is completely suppressed by TAK1K63W; however, this effect is masked (or counteracted) by apoptosis-mediated induction of these chemokines. Remarkably, apoptotic events cannot rescue the strong reduction of constitutive chemokine expression that is found in TAK1K63W-expressing cells (Fig. 5, compare A with B). The induction of genes by apoptosis is likely to be specific for distinct inflammatory genes, because mRNA levels of housekeeping genes decrease in TNF-stimulated TAK1K63W-expressing cells, an effect that was prevented by Z-VAD-fmk (supplemental Table II). The complete set of data for the experiments shown in Fig. 5 is provided in supplemental Table II.

Additional experiments with the specific PI3K inhibitor wortmannin revealed that none of the TNF-induced genes detected by the oligonucleotide microarray analysis was significantly suppressed at doses of wortmannin (100 and 1000 nM) that completely inhibited basal and inducible phosphorylation of AKT (Fig. 6). The latter experiments further support a specific role of TAK1 but not of PI3K-AKT in the regulation of TNF-induced inflammatory genes. These results strongly argue for a role of the PI3K-AKT signaling pathway in other TNF responses and heighten the likelihood that PI3K-AKT contributes to the regulation of TAK1-independent genes.
Validation of Key Gene Expression Changes Detected in Microarray Studies by Northern Blot and Chromatin Immunoprecipitation—

To validate the results of the microarray hybridization studies, Northern blot analysis was performed on four of the inflammatory genes most strongly induced by TNF using RNA prepared from cells at different times after TNF addition. Time course Northern blot experiments revealed strong and rapid induction of ccl2, ccl7, saa3, and slpi mRNA in response to TNF and verified inhibition of this induction in cells expressing TAK1K63W (Fig. 7A). At the exposure times chosen, no basal mRNA expression of these genes was detected on Northern blot analysis of TAK1K63W-expressing cells and very little
was detected in the control cells. TNF induced very strong signals in the control cells but not in the dominant negative TAK1-expressing cells, confirming the large difference in mRNA expression levels between those seen in the microarray experiments shown in Figs. 4 and 5. The steady-state differences in mRNA levels seen in the microarray analysis could be attributed to increased transcription or to post-transcriptional effects (1). To determine whether TAK1K63W affected mRNA transcriptional regulation, we measured the recruitment of RNA polymerase II to the region around the known transcriptional start sites of the promoters of \textit{ccl2}, \textit{ccl7}, \textit{saa3}, and \textit{slpi} in unstimulated cells and in response to TNF (Fig. 7B). All of the promoters showed a basal level of RNA polymerase II binding that was stimulated 1.5–4.5-fold by 90 min of TNF stimulation (Fig. 7B). Very interestingly, basal and inducible recruitment of RNA polymerase II to these promoters is strongly inhibited by the TAK1K63W mutant (Fig. 7B), indicating that TAK1 activity regulates expression of downstream genes directly at the transcription machinery level.

To further analyze the mechanism by which TAK1K63 strongly suppresses basal and stimulated promoter occupancy by RNA polymerase II, we focused on transcriptional regula-
The proximal 2.8-kb upstream region contains NFκB sites identified by DNase I-hypersensitive site mapping (Fig. 8A), located adjacent to each other on mouse chromosome 11 (Fig. 9A). The ccl2 gene locus has previously been reported to contain three proximal and two distal 5'-regulatory regions that were identified by DNase I hypersensitive site mapping (Fig. 8B) (31). The proximal 2.8-kb upstream region contains NFκB and AP-1 sites (36, 37), whereas the distal region spans −6 kb (−14 to −20 kb) and contributes to TNF-dependent ccl2 transcription by unknown elements (31). Transfection experiments using constructs of these regions and luciferase reporters revealed that the additional region 20 kb upstream of the transcriptional start site was weakly activated by TNF and induction by unknown elements (31). Transfection experiments using constructs of these regions and luciferase reporters revealed that the additional region 20 kb upstream of the transcriptional start site was weakly activated by TNF and induction by unknown elements (31). The inflammatory response is a complex process that is characterized by the induction (or repression) of hundreds of genes in a wide variety of cell types. In its acute form, inflammation can be beneficial because it is the first-line innate immune defense that limits and repairs tissue damage in response to injury and infection. However, in its chronic form, inflammation turns into a destructive tissue-destroying process. No curative treatment for chronic inflammation is available, but therapies directed at either TNF or IL-1 have been successful in ameliorating this condition (38, 39). However, these agents neutralize extracellular TNF or IL-1 and hence block the complete cellular response to these cytokines, not all of which are deleterious processes. It is evident that novel strategies to modulate inflammation must be developed and targeted to the specific induction or suppression of subsets of products of IL-1 or TNF target genes to control unwanted or desired effects of inflammation (38).
in control cells, 263 only in TAK1K63W-expressing cells, and regulated genes, still identified 506 TNF-regulated genes only.

Press the activation of PI3K.

dominant negative TAK1 because its expression does not sup-

TNF. Importantly, these pathways are specific targets of the 

ways as well as to the apoptosis-executing program.

It follows that blocking signaling molecules that (i) have the 

capacity to control both the activation of NfkB and MAPK pathways simultaneously and that (ii) are activated by only a subset of all the ligands that stimulate MAPK and NfkB should be advantageous over the strategies outlined above.

We have tested this hypothesis by focusing on the conse-
quen ces of inhibiting the activity of TAK1. As shown here, retroviral transduction of fibroblasts with a kinase-dead dom-
ants negative mutant of TAK1, TAK1K63W, potently inhibits the NfkB, JNK, and p38 activation typically induced by IL-1 or TNF. Importantly, these pathways are specific targets of the dominant negative TAK1 because its expression does not suppress the activation of PI3K.

These cell lines do not exhibit any phenotypic change in the absence of proinflammatory cytokine treatment. However, unlike wild-type NIH3T3 cells, they rapidly undergo apo-
poptosis if treated with TNF. It is known that, in cells deficient in I KK or NfkB subunits, the lack of NfkB-dependent expres-
sion of anti-apoptotic genes such as inhibitors of apo-
poptosis contributes to apoptosis (7, 45). However, these cells also have a prolonged JNK activation following TNF treat-
ment and JNK was shown to promote TNF-induced apoptosis (46–49).

Our results extend these observations by showing that apoptosis can also occur if both NfkB and JNK are blocked. Thus, the results presented in Figs. 1 and 2 suggest that, in fibroblasts, TAK1 has a specific role in coupling activated TNF receptors to the NfkB, JNK, and p38 MAPK pathways as well as to the apoptosis-executing program.

Therefore, we used the cell lines characterized in this study to identify TAK1 target genes by a combination of high-density cDNA and low-density oligonucleotide microarrays. We found that expression of a large number of genes was up-regulated or down-regulated at least 2-fold by TNF in control cells. Filtering the same experiments more stringently, i.e., for at least 3-fold regulated genes, still identified 506 TNF-regulated genes only in control cells, 263 only in TAK1K63W-expressing cells, and 203 that were regulated by TNF in both cell types (data not shown). The number of TNF-regulated genes varied widely in different published microarray studies (50–54). However, in each report, a different microarray platform or cell type was used, precluding a direct comparison of our estimate of the number of TNF-regulated genes with those of these studies. However, in agreement with our observations, others (51, 53, 54) have also found several hundred genes to be regulated by TNF. ~50% of the 1500 TNF-inducible genes were compromised by TAK1K63W, but we also found a surprisingly large number of genes that were still regulated by TNF, even in the presence of TAK1K63W expression. We also found genes that were induced by TAK1K63W such as c-fos, gadd45β/γ, and others. Most impressively, we identified a number of genes whose expression was very strongly suppressed by TAK1K63W. This suppression was most complete for ccl2 and ccl7. As shown in Fig. 5, A and B, ccl2, ccl7, and several other important chemokines show severely depressed basal levels of expression when TAK1 activity was compromised by expres-
sion of dominant negative TAK1K63W. Nonetheless, these genes are still up-regulated severalfold by TNF treatment, although these levels rarely reach even the basal expression level in control cells (Fig. 5). This TNF-induced stimulation of expression in TAK1K63W cells is almost completely abrogated by the caspase inhibitor Z-VAD-fmk.

The data presented in Fig. 5 also show that TNF-inducible genes are affected to different extents by the TAK1 mutant, indicating substantial quantitative differences in the regula-
tion of gene expression by TAK1-dependent signaling path-
ways. For example, the expression of ccl2 and ccl7 is strikingly dependent on TAK1, whereas the suppression of c-Jun only becomes apparent if cells are treated with Z-VAD-fmk. Collectively, these results demonstrate the importance of separately investigating basal and inducible mRNA levels in microarray experiments in situations in which multiple cellular pathways are perturbed.

One interpretation of these results is that the attenuated basal transcription level of ccl2 and ccl7 reflects the lack of certain components of the basal transcription machinery. How-

er, the components that are there are sufficient to respond to TNF stimulation by increasing the transcription rate of these chemokine genes. One or more of these components appears to require caspase activity for its activation, a situation that cannot be appreciated in control cells, which have a much higher basal expression of these genes. Because this effect is post-
transcriptional and post-translational, it is unlikely to be dis-
covered by the profiling of gene expression at the mRNA level as utilized in this study and warrants further investigation by proteomic methods.

We initially envisaged to use the microarray results to un-
cover specific mechanisms of TAK1 target gene activation by a groupwise comparison of the sequences of all of the genes that are not affected or that are even induced by TAK1K63W with those genes that are strongly suppressed. Such a comprehen-

sive approach ideally requires identification of common regu-

latory regions of genes showing similar regulation patterns by bioinformatical methods followed by experimental verification. The number and heterogeneity of the TAK1-dependent genes and the complexity of available information on gene structures of ccl2, ccl7, saa3, and slpi made this is a formidable task, which was eventually beyond the scope of this study. Similar difficulties have been noticed and discussed in depth in some recent reviews (55, 56). Therefore, we limited our further anal-

ysis to the most strongly suppressed ccl2 and ccl7 genes.

Our data show that TAK1 is required for maintaining con-
stitutive as well as inducible mRNA synthesis of ccl2 and ccl7 by controlling the recruitment of RNA polymerase II. Similar to
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many other chemokines, ccl2 and ccl7 are induced with the same intensity and kinetics by TNF (Fig. 6). It has long been known that chemokine genes are clustered on chromosomes 5 and 11 for man and mouse, respectively. The availability of previously characterized large fragments of upstream regulatory regions of the mouse ccl2 locus enabled us to show that TAK1K63W affects not only the immediate upstream proximal region of the ccl2 promoter but that it also suppresses activity of a 20-kb upstream regulatory region when fused to a minimal ccl2 promoter fragment. The upstream region spans at least 6 kb and may represent a locus control region required for the effective expression of ccl2, ccl7, and other chemokines genes. Our data support the regulatory role of this region, which was previously suggested by the appearance of DNase I-hypersensitive sites in response to TNF (31). Further insight into this mechanism requires the identification of the cis-elements in sensitive sites in response to TNF (31). Further insight into this mechanism requires the identification of the cis-elements in sensitive sites in response to TNF (31). Further insight into this mechanism requires the identification of the cis-elements in sensitive sites in response to TNF (31).

Our data support the regulatory role of this region, which was clearly recapitulated the suppression of TAK1K63W. In contrast to observations in human cells (59, 60) our study)

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