Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 340 (2005) 255 – 264

VIROLOGY

www.elsevier.com/locate/yviro

The MCV MC159 protein inhibits late, but not early, events of TNF-α-induced NF-κB activation

Lyre Espada Murao[1], Joanna L. Shisler*

Department of Microbiology, College of Medicine, University Of Illinois, C222 CLSL, 601 S. Goodwin Avenue, Urbana, IL 61801, USA

Received 11 May 2005; returned to author for revision 6 June 2005; accepted 22 June 2005
Available online 25 July 2005

Abstract

Tumor necrosis factor (TNF-α) triggers biphasic activation of the NF-κB transcriptional regulator. This process consists of an initial, IκBα-mediated transient phase and a later, persistent phase dependent on IκBβ degradation. To presumably interfere with the fulfillment of this immunity-associated event in cells infected with the molluscum contagiosum virus (MCV), this pathogen produces the intracellular MC159 protein. To define the mode of action of MC159, the impact of TNF-α on HEK 293T cells ectopically expressing the MC159 protein was examined. In this regard, TNF-α-induced expression of an NF-κB-regulated luciferase reporter gene was partially inhibited by the MC159 protein. This ability was attributed to blockage of the persistent phase of TNF-α-induced NF-κB activation for the following reasons: (1) the initial phase of NF-κB transcriptional activation was not affected by the MC159 protein; (2) the MC159 protein inhibited TNF-α-directed degradation of IκBβ, but not IκBα; and (3) expression of the late NF-κB-regulated cell genes, TNF-α and CCL2, was decreased in the presence of the MC159 protein while transcription of the early NF-κB-regulated cell gene, CXCL1, was not altered. Previously reported MC159–RIP interactions appear to be irrelevant for the MC159 inhibitory function. In contrast, MC159–TRAF2 associations are more relevant for inhibitory function since mutant MC159 proteins unable to bind TRAF2 also cannot inhibit TNF-mediated NF-κB activation. In vivo, the MC159 protein may act to prolong virus survival by preventing the infected cell from responding to TNF-α, ultimately preventing the cellular production of proinflammatory and immunoattractant molecules.

Keywords: MC159; MCV; NF-κB; TNF-α; v-FLIP; IκBα; IκBβ; RIP; TRAF2

Introduction

Tumor necrosis factor-α (TNF-α) is a pluripotent cytokine that activates the NF-κB signal transduction pathway in a biphasic, temporal manner (Hoffmann et al., 2002; Schmidt et al., 2003). Initially, binding of TNF-α to TNF-Receptor I (TNF-RI) triggers TRADD (TNF-RI-Associated death domain), RIP (receptor-interacting protein) and TRAF2 (TNF-receptor-associated factor) migration to the receptor, forming a signalsome that subsequently recruits MEKK (Mitogen-activated protein kinase kinase) proteins (Chen and Goeddel, 2002; Yang et al., 2001). The MEKK proteins in turn activate the I kappa kinase (IKK) complex, and the resultant complexes interact with specific IκB/NF-κB complexes (Schmidt et al., 2003). The rapid association of MEKK3 with IKK and IκBα/NF-κB complexes enables phosphorylation and degradation of the NF-κB-associated IκBα molecule, resulting in NF-κB nuclear translocation and subsequent transcriptional activation of NF-κB-regulated genes. This transient phase is quickly terminated because the released NF-κB induces IκBα production and nascent IκBα binds to and inhibits NF-κB. However, in the continued presence of TNF-α, MEKK2 associates with IKK, and forms IκBβ/NF-κB/IKK/MEKK2 complexes. During this persistent activation phase IκBβ is degraded

* Corresponding author. Fax: +1 217 244 6697.
E-mail addresses: lyremurao@yahoo.com (L.E. Murao), jshisler@uiuc.edu (J.L. Shisler).
[1] Current address: 178 Sparrow Street, Ecoland Phase II, Matina, Davao City 8000, Philippines.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.06.036

and this newly released NF-κB population translocates to the nucleus. As would be expected from this biphasic activation, some "early" NF-κB-regulated genes are expressed rapidly after TNF-α treatment, while other "intermediate" and "late" genes are transcribed only after persistent incubation with TNF-α (Hoffmann et al., 2002, 2003; Zhou et al., 2003).

The MC159 product of the molluscum contagiosum virus (MCV) was previously shown to inhibit NF-κB activation induced by the over-expression of TNF-RI molecules (Chaudhary et al., 1999). The goal of this study was to determine the effect of the MC159 protein on the transient and persistent phases of TNF-α-induced NF-κB activation. We examined TNF-α-mediated IκBα and IκBβ degradation, NF-κB transcriptional activation and NF-κB-mediated gene transcription in 293T cells ectopically expressing the MC159 gene product. We also examined the relationship between MC159-RIP and MC159–TRAF2 associations and their role in inhibiting TNF-α-mediated signaling.

Results

MC159 proteins inhibit TNF-α-induced expression of a luciferase reporter gene

NF-κB activation induced via over-expression of death receptors was previously shown to be inhibited by MC159 expression (Chaudhary et al., 1999). To use a model system more closely mimicking the physiological environment, we chose to activate TNF-RI signaling events by incubating cells with TNF-α. This system was previously utilized to prove that MC159 inhibited death-receptor-induced apoptosis (Bertin et al., 1997). MC159 inhibitory function was assayed by comparing NF-κB-driven firefly luciferase production in TNF-α-treated and untreated cells. TNF-α-induced luciferase activity, which correlates with NF-κB activation, was significantly lower in MC159-expressing cells versus vector-transfected cells at all time points tested ($P < 0.006$) (Fig. 1A). Luciferase activity in MC159 expressing cells was 50, 75 and 90% lower than pCI-transfected cells after 6, 12, and 24 h of TNF-α treatment, respectively. MC159 protein levels were similar in all cells, as determined by immunoblotting extracts for the presence of MC159 (Fig. 1). In some assays, luciferase activity was slightly increased in untreated, MC159-expressing cells (13- to 14-fold higher) as compared to untreated, pCI-transfected cells (Fig. 1). This activating effect was marginal compared to TNF-α induction. The level of luciferase activity returned to that detected in vector-transfected cells when MC159-expressing cells were incubated for longer times (data not shown). Luciferase activities were also significantly lower ($P < 0.004$) in MC159-expressing cells treated with either 1, 5 or 10 ng TNF-α/ml for 12 h (Fig. 1B).

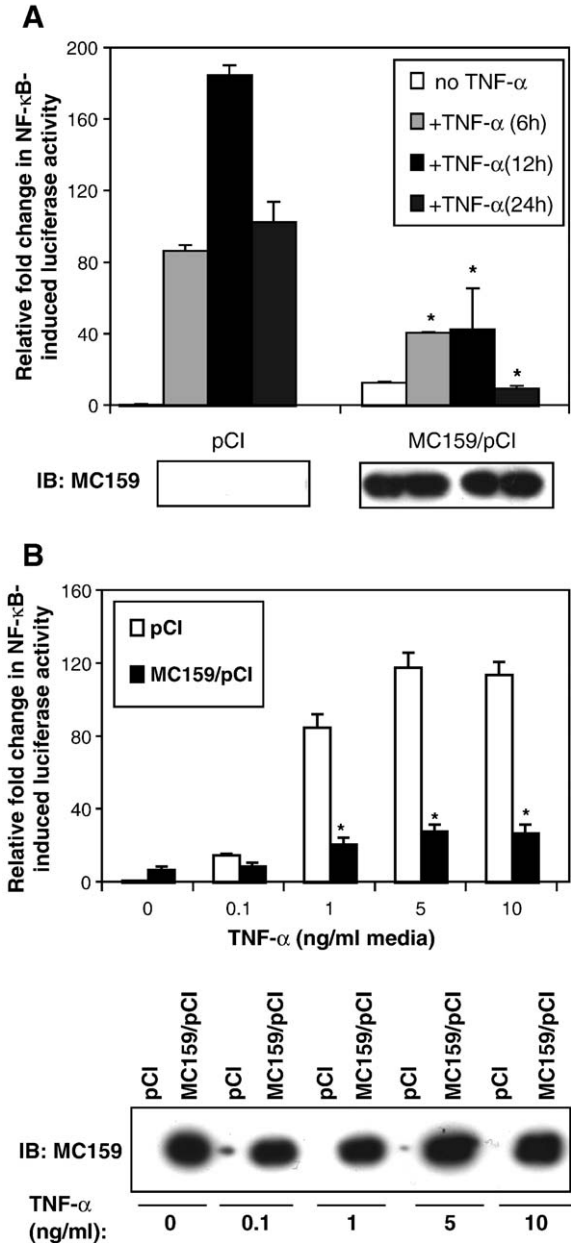

Fig. 1. The effect of MC159 expression on TNF-α-induced NF-κB activation. 293T cells were co-transfected with a plasmid that has a firefly luciferase gene under the control of an NF-κB promoter (pNF-κB*luc*), a plasmid with a sea pansy luciferase gene expressed constitutively (pRL-null) and either a plasmid expressing MC159 (MC159/pCI) or empty vector (pCI) and incubated for 24 h. (A) Transfected cells were treated with media absent for or containing TNF-α (10 ng/ml) for 6, 12 or 24 h. Cells were collected and lysed, and luciferase activities were measured. An asterisk (*) indicates statistically significant inhibition of luciferase activity ($P < 0.006$) in MC159/pCI-transfected cells versus pCI-transfected cells. (B) Transfected cells were incubated with media containing 0–10 ng TNF-α/ml media for 12 h. Cells were collected and lysed, and luciferase activities were measured. Statistical significant inhibition of luciferase activity ($P < 0.004$) in MC159/pCI- versus pCI-transfected cells is denoted by an asterisk (*). For all experiments, a portion of each lysate was also analyzed for MC159 expression by probing immunoblots (IB) with anti-MC159 antisera (see IB below each graph).

MC159 protein expression does not inhibit the initial stage of NF-κB transcriptional activation

To determine the mechanism of NF-κB inactivation by MC159, NF-κB transcriptional activation (and therefore nuclear translocation), was examined in pCI- and MC159/pCI-transfected cells via electromobility shift assays (Fig. 2). A mobility-shifted band (indicative of active NF-κB) was prominent when vector-transfected cells were incubated with TNF-α for 30–90 min (Fig. 2A). The band intensity decreased during longer TNF-α incubation times (105–120 min), and reappeared again at 150 and 210 min. Likewise, a band with the same altered mobility was also detected when assaying MC159-expressing cells treated with TNF-α for 30–90 min and continued to be detected until 180 min of TNF-α treatment, indicating that MC159 did not inhibit the initial TNF-α-induced NF-κB transcriptional activation (Fig. 2A). The intensity of the shifted band started to decrease at 150 min of TNF-α treatment and was undetectable by 210 min, suggesting that MC159 inhibited the later phase of TNF-induced NF-κB transcriptional activation.

To confirm that the p65 and p50 subunits of NF-κB were responsible for retarding the migration of the radiolabeled oligonucleotide in these assays, extracts from TNF-α-treated, vector-transfected cells were incubated with either anti-p65 or anti-p50 antisera (Fig. 2B). In comparison to samples lacking the antibodies, the NF-κB-containing band was decreased in intensity in reactions containing either anti-p65 or anti-p50 antisera. In general, the absence of a super-shifted band is evidence (albeit indirect) that anti-p65 has recognized and bound to p65 proteins. Additionally, a super-shifted band was detectable in reactions containing anti-p50 antisera (Fig. 2B), confirming that the p50 subunit is one of the major NF-κB species active during TNF treatment. In contrast, anti-p52 or anti-c-Rel antisera did not alter the mobility or change the intensity of the NF-κB-containing band (Fig. 2B). Similar results were observed when nuclear proteins from MC159-expressing cells were tested (Fig. 2B), indicating that MC159 expression did not

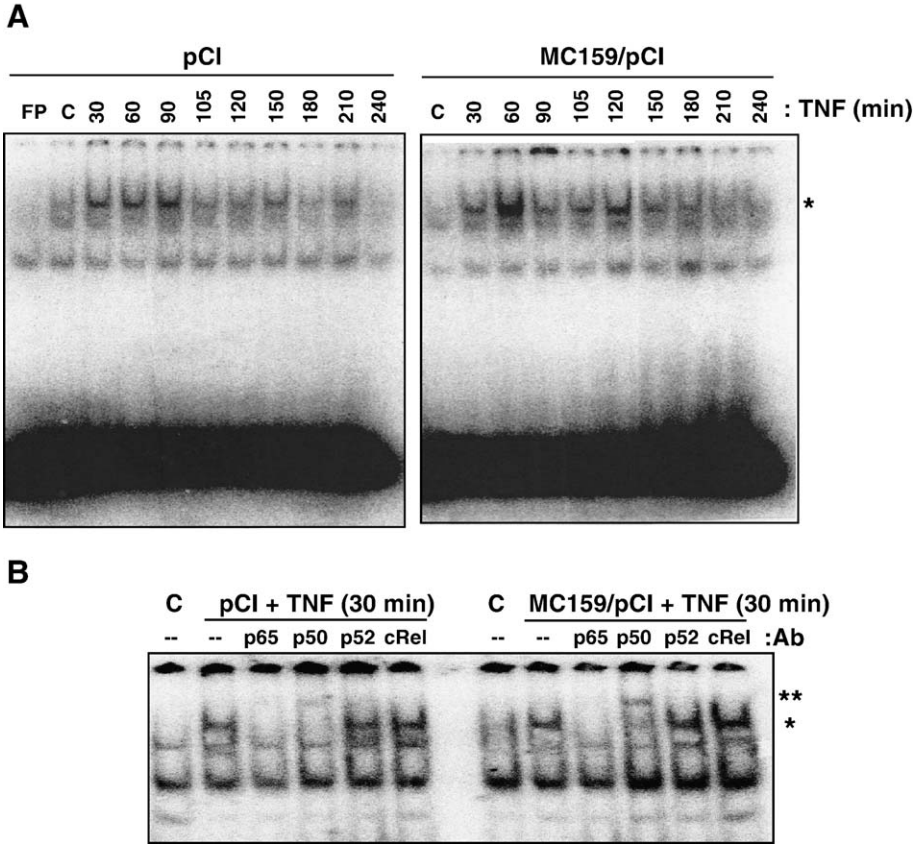

Fig. 2. TNF-α-induced nuclear localization of NF-κB. 293T cells were transfected with either pCI or MC159/pCI. At 24 h post-transfection, cells were treated with medium alone ("C") or containing TNF-α (10 ng/ml) for the times indicated. (A) Cells were harvested and 2 μg of nuclear-extracted proteins from each sample were incubated with ^{32}P-radiolabeled oligonucleotide containing consensus NF-κB binding sites and assayed via an electromobility shift assay. Mobility-shifted bands containing NF-κB are indicated by an asterisk (*). Analysis of a reaction containing only unbound radiolabeled probe is indicated in lane "FP." (B) Nuclear extracts from untreated cells ("C") or cells treated with TNF-α for 30 min were pre-incubated with the indicated antibodies ("Ab") or no antisera ("–") for 10 min at room temperature before the addition of radiolabeled oligonucleotides. The single asterisk (*) represents the mobility-shifted NF-κB complexed with radiolabeled oligonucleotide while the double asterisk (**) represents the super-shifted complex of NF-κB bound with radiolabeled oligonucleotides and anti-NF-κB antisera.

substantially alter the transcriptional activation of different NF-κB subunits.

The MC159 protein inhibits TNF-α-mediated IκBβ degradation, but not IκBα degradation

Results from the electromobility shift assay appeared to contradict those from the luciferase assay: MC159 expression did not alter early TNF-α induced NF-κB transcriptional activation although it decreased NF-κB-controlled firefly luciferase expression and inhibited later phases of TNF-α induced NF-κB transcriptional activation. Since the two phases of TNF-α induced NF-κB activation are differentially regulated (Schmidt et al., 2003; Thompson et al., 1995), one hypothesis was that the MC159 protein did not affect IκBα-mediated transient NF-κB activation, but instead inhibited the IκBβ-mediated persistent activation of NF-κB.

To more accurately assess the effect of MC159 gene expression on the transient and persistent NF-κB activation phases, we examined cellular IκBα and IκBβ levels, respectively, in cells treated with TNF-α in the presence or absence of MC159 proteins (Fig. 3). After 30 min exposure to TNF-α, the amount of cellular IκBα decreased dramatically in both pCI- and MC159/pCI-transfected cells (Fig. 3A). Nascent IκBα protein was detected in cells incubated with TNF-α for 60–120 min, in agreement with a previous report (Schmidt et al., 2003).

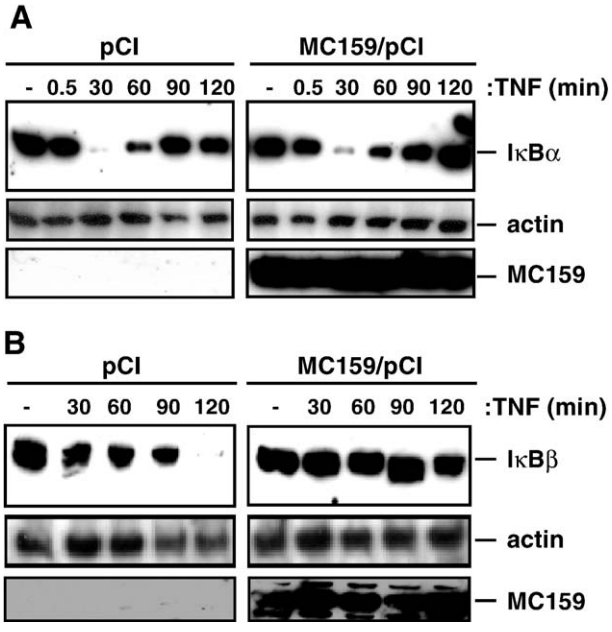

Fig. 3. IκBα and IκBβ levels in TNF-α-treated cells. 293T cells were transfected with either pCI or MC159/pCI. At 24 h post-transfection, cells were incubated with media alone or media containing TNF-α (10 ng/ml) for the indicated times. Cells were collected and lysed. Equal amount of cytoplasmic extracts were electrophoretically separated by 12% SDS-PAGE and analyzed by probing immunoblots with either (A) anti-IκBα or (B) anti-IκBβ antisera. A separate set of identical blots was incubated with anti-MC159 and with anti-actin antibodies.

TNF-α-induced IκBβ protein degradation occurs later than IκBα degradation (Schmidt et al., 2003; Thompson et al., 1995). Thus, as expected, IκBβ levels were slightly decreased in vector-transfected cells incubated with TNF-α for 60 min, and was undetectable by 120 min post-treatment (Fig. 3B). This correlates with the detection of a faint mobility-shifted band in nuclear extracts from pCI-transfected cells incubated with TNF for 150–210 min (Fig. 2A). In contrast, IκBβ protein levels in MC159-expressing cells remained constant at all times (Fig. 3B). This correlates with the decreasing intensity of the mobility-shifted band in nuclear extracts from MC159-transfected cells incubated with TNF-α for 150–240 min (Fig. 2A). It should be noted that IκB protein degradation was not due to global protein degradation being induced by TNF-α in the absence of the MC159 protein as the relative amount of actin remained constant in both types of transfected cells (Fig. 3).

MC159 inhibits activation of genes expressed later after TNF-α treatment

As a result of biphasic NF-κB activation, TNF-α induces the expression of some genes immediately, while that of others is upregulated later (Hoffmann et al., 2002, 2003; Zhou et al., 2003). Since the MC159 protein prevented IκBβ degradation, but did not alter IκBα degradation, we hypothesized that MC159 would not affect early gene transcription, but would decrease the activation of genes expressed late after exposure to TNF-α. Accordingly, we screened for the presence of mRNA from genes previously characterized as either early or later responders to TNF-α exposure (Hoffmann et al., 2002, 2003; Zhou et al., 2003) by using semi-quantitative reverse transcriptase PCR (RT-PCR).

The CXCL1 gene is expressed soon after TNF-α treatment of cells (Hoffmann et al., 2002, 2003; Zhou et al., 2003) and CXCL1 cDNA was easily detected when pCI- and MC159/pCI-transfected cells were treated with TNF-α for 1 h (Fig. 4A), indicating that the CXCL1 gene had been transcribed. GAPDH cDNA was co-amplified in each PCR reaction, verifying that cells transcribe CXCL1 mRNA only in response to TNF-α. After 8 h TNF-α treatment, CXCL1 cDNA was similar to the level amplified in pCI-transfected cells treated with TNF-α for 1 h. CXCL-1 cDNA was slightly decreased in MC159-expressing cells treated with TNF-α for 8 h.

TNF-α and CCL-2 genes are expressed at later times in response to TNF-α-induced NF-κB activation (Hoffmann et al., 2002, 2003; Zhou et al., 2003). Accordingly, TNF-α and CCL-2 PCR products were undetectable in vector- and MC159/pCI-transfected cells incubated with TNF-α from 0 to 1 h, but appeared after 8 h (Figs. 4B and C). In comparison to pCI-transfected cells, the TNF-α amplicon was less abundant (Fig. 4B) in MC159-expressing cells that were incubated with TNF-α for 8 h. Similarly, CCL-2 PCR products were decreased in response to TNF-α

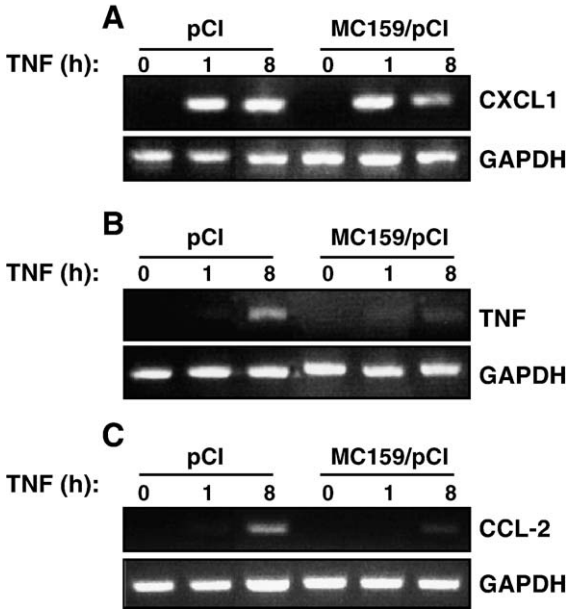

Fig. 4. TNF-α-induced transcription of early and intermediate NF-κB-regulated host cell genes. 293T cells were transfected with either plasmid pCI or MC159/pCI. At 24 h post-transfection, cells were incubated with media alone or media containing TNF-α (10 ng/ml). Cells were collected and harvested after either 0, 1 or 8 h after TNF-α treatment, and RNA was extracted and reverse transcribed. The resulting cDNA was incubated with primers specific for the (A) CXCL1, (B) TNF-α or (C) CCL-2 genes, as well as primers to the GAPDH housekeeping gene and subjected to PCR amplification. PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

in MC159-expressing samples versus vector-transfected samples (Fig. 4C).

The MC159 protein does not inhibit RIP-induced NF-κB activation

The RIP accessory molecule is important in recruiting MEKK3 to the TNF-RI complex (Blonska et al., 2004; Yang et al., 2001), suggesting that RIP controls the early phase of TNF-mediated NF-κB activation by specifically activating MEKK3. Our data indicated that MC159 did not prevent the MEKK3-regulated early phase of NF-κB activation, suggesting that previously reported RIP–MC159 interactions were not biologically relevant (Chaudhary et al., 1999). To test this hypothesis, we assayed the ability of MC159 to inhibit NF-κB activated by RIP over-expression. Luciferase activity was not significantly lower when cells were co-transfected with 500 ng of plasmid pRIP and MC159/pCI versus pRIP and pCI (Fig. 5). Luciferase levels were higher in MC159-expressing cells than in control cells when cells were transfected with lower amounts of pRIP. Since MC159 over-expression alone induced luciferase activity, then higher luciferase levels detected in MC159/pCI-pRIP co-transfected cells were additive. As expected, MC159 protein expression was equal in all MC159/pCI-transfected cells and RIP expression increased as the amount of pRIP present in transfection reactions increased (Fig. 5, immunoblots).

MC159–TRAF2 binding correlates with the MC159 inhibitory function

MC159–TRAF2 interactions were also reported previously (Chaudhary et al., 1999). TRAF2 over-expression via ectopic expression weakly induces NF-κB activation, even when 2000 ng of a pTRAF2 plasmid is transfected into cells (data not shown). Therefore, instead of using NF-κB luciferase reporter assays to investigate the role of TRAF2 in the inhibitory function of MC159, we analyzed mutant MC159 proteins missing either the first or second death effector domain (DED) (Garvey et al., 2002a) for their ability to (1) bind to TRAF2 molecules, and (2) inhibit TNF-induced luciferase activity.

Cells were co-transfected with plasmid pTRAF2 and plasmids containing either the wild type or mutated MC159 ORFs (pMC159A and pMC159B). pMC159A expresses the MC159A mutant protein, which is missing the second DED while pMC159B codes for the MC159B protein, which lacks the first DED (Garvey et al., 2002a). TRAF2 proteins were immunoprecipitated from cell lysates and the presence of MC159 was assayed for by immunoblotting the immunoprecipitated samples (Fig. 6A). Both the 25-kDa wild type and 15-kDa MC159A proteins were readily detected in immunoprecipitates. In contrast, very little MC159B proteins associated with TRAF2. The TRAF2

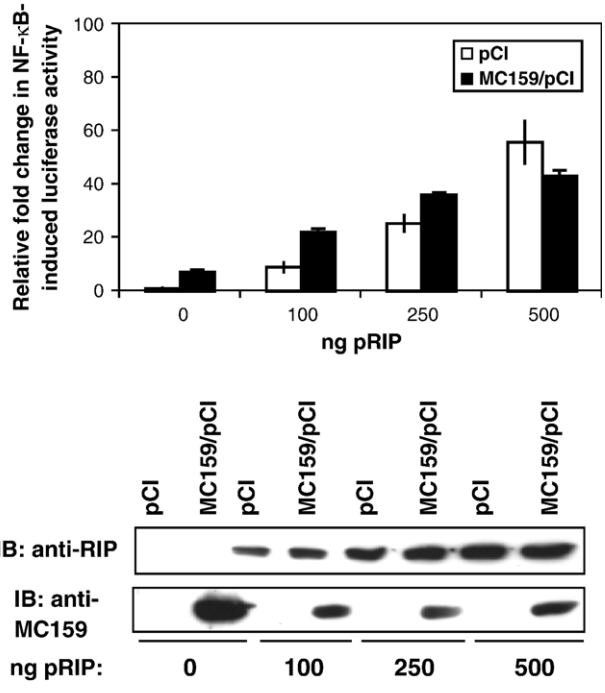

Fig. 5. The effect of MC159 protein expression on RIP-mediated NF-κB activation. 293T cells were transfected with 450 ng pNF-κB*luc*, 50 ng pRLnull, 0–500 ng pRIP and 1000 ng of either pCI or MC159/pCI. At 24 h post-transfection, cells were collected and lysed, and luciferase activities were measured. A portion of each lysate was analyzed by using immunoblotting ("IB"). Immunoblots were probed with either anti-RIP or anti-MC159 antisera for detection of RIP or MC159 proteins, respectively.

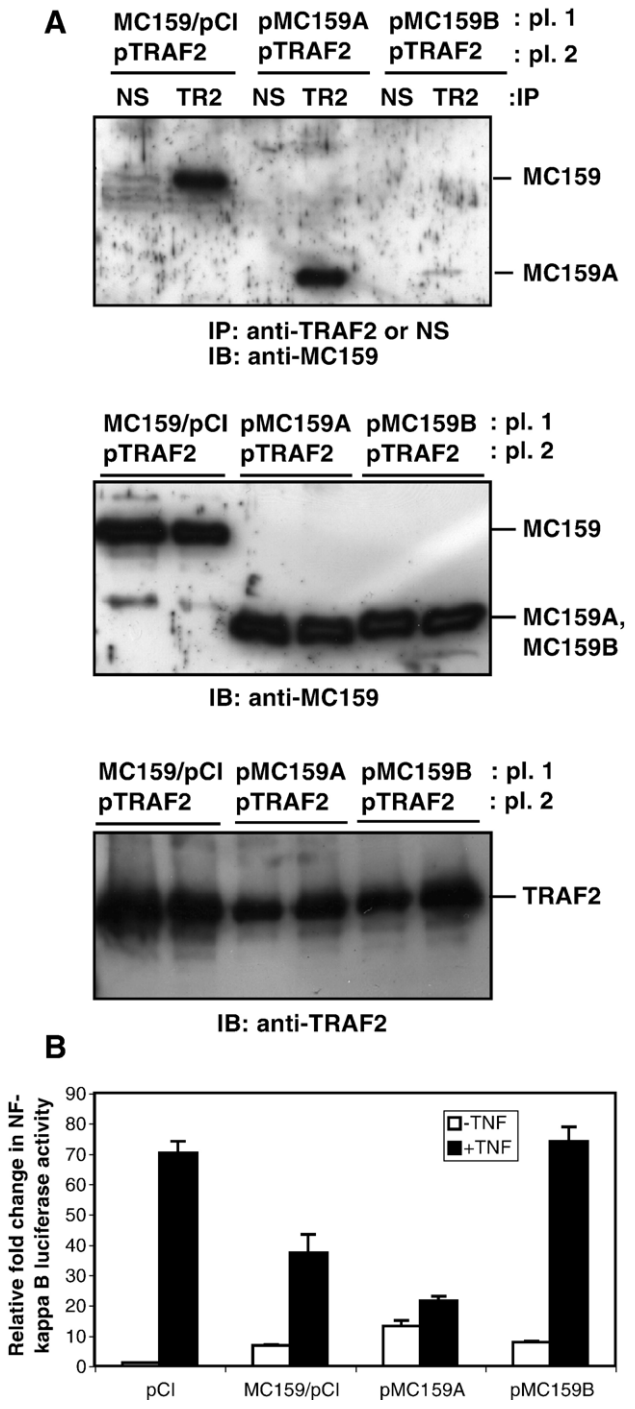

Fig. 6. The effect of mutant MC159 protein expression on TRAF2 binding and TNF-induced NF-κB activation. (A) Cells were transfected with 500 ng pTRAF2 and 500 ng of either MC159/pCI, pMC159A or pMC159B. 24 h later, cells were collected and lysates were incubated with either anti-TRAF2 (TR2) or rabbit IgG (NS) as a negative control. Immunoprecipitated samples were analyzed by using immunoblotting for MC159 proteins (upper panel). A portion of each pre-immunoprecipitated lysate sample was analyzed for either MC159 or TRAF2 expression by using immunoblotting (middle and lower panels, respectively). (B) 293T cells were transfected with 450 ng pNF-κB*luc*, 50 ng pRLnull, 500 ng of either pCI, MC159/pCI, pMC159A or pMC159B for 24 h. Transfected cells were treated with media absent for or containing 10 ng/ml TNF-α for 12 h. Cells were collected and lysed, and luciferase activities were measured.

and MC159 proteins were expressed equally, ruling out the possibility that the lack of MC159B–TRAF2 interactions was due to low protein expression (Fig. 6A, middle and lower panels). These interactions are specific, as confirmed by the absence of wild type and mutant MC159 proteins in immunoprecipitation reactions using a non-specific (NS) antibody.

Next, TNF-α-induced NF-κB luciferase activity in cells expressing the mutant MC159 proteins was compared to cells expressing wild-type MC159 products. TNF-induced luciferase activity in MC159A-expressing cells was approximately 2.5-fold lower than pCI-transfected cells, similar to levels in cells expressing wild-type MC159 proteins (Fig. 6B). MC159B molecule expression did not inhibit luciferase activity since luciferase levels in pMC159B- and pCI-transfected cells were similar (Fig. 6B).

Discussion

TNF-α is a powerful proinflammatory cytokine that mediates anti-viral effects. Many viruses, including poxviruses, inhibit TNF-α signaling to enhance survival (Hiscott et al., 2001; Seet et al., 2003). Here, we present data that the MCV MC159 protein prevents TNF-α-induced IκBβ degradation and consequently the NF-κB transcriptional activation and NF-κB-mediated expression of cellular genes that are initiated later in response to TNF-α. Moreover, we found that the MC159 protein did not prevent IκBα degradation, correlating with its inability to inhibit transient NF-κB transcriptional activation, or inhibit the upregulation of transcription of early host genes. Thus, MC159 appears to utilize a novel mechanism, specifically targeting the persistent phase of TNF-α-induced NF-κB activation to dampen later TNF-α-mediated signaling events.

TNF-RI-mediated NF-κB activation is dependent on the formation of a TRADD-RIP-TRAF2 signalsome that in turn activates the IKK complex, resulting in IκBα degradation and NF-κB activation (Chen and Goeddel, 2002). Because MC159 co-immunoprecipitates with RIP (Chaudhary et al., 1999), it was hypothesized that MC159 interacts with this protein to inhibit signalsome formation and subsequent downstream NF-κB activation events (i.e., IKK activation, IκBα degradation and early NF-κB transcriptional activation). However, the expression of MC159 did not inhibit these events (Figs. 2 and 3A), suggesting that the MC159–RIP interactions are not biologically relevant. In support of this idea, we observed that MC159 did not prevent NF-κB activation induced by RIP over-expression (Fig. 5). A report from Chan et al. (2003) showing that MC159 does not interfere with RIP migration to the TNF–RI signalsome also bolsters the hypothesis that previously reported MC159–RIP interactions are not important for MC159 inhibitory function.

MC159 possesses two DEDs (Senkevich et al., 1996). These motifs are important for protein–protein interactions and were originally predicted to be important for MC159 to

associate with other DED-or death domain-containing proteins (Senkevich et al., 1996, 1997). Indeed, MC159–FADD and MC159–procaspase-8 interactions were originally thought to be critical for MC159's ability to inhibit TNF-mediated apoptosis (Bertin et al., 1997). A more detailed analysis of MC159 reveals that anti-apoptosis activity does not correlate with MC159–FADD or MC159–procaspase-8 interactions (Garvey et al., 2002b). Similarly, we have found that RIP–MC159 interactions are not predictive of a MC159 mechanism for inhibiting NF-κB activation.

The MC159 protein also inhibits NF-κB activation mediated through PKR (Gil et al., 2001) and MyD88 over-expression (data not shown). Since TRAF proteins are critical accessory molecules common among the PKR, Toll-like receptor and TNF-RI NF-κB activation pathways, one likely inhibitory mechanism for the MC159 protein is to inhibit TRAF activity. In addition to co-immunoprecipitation data demonstrating MC159–TRAF2 interactions (Chaudhary et al., 1999), we observed that the mutant MC159A protein inhibited TNF-induced NF-κB activation and associated with TRAF2. In contrast, the mutant MC159B protein, which did not bind TRAF2, did not inhibit TNF-induced luciferase activity. These data implied that MC159–TRAF2 interactions are critical for the inhibitory function of MC159. Further, the MC159 cellular homolog cFLIP(L) requires TRAF2 interactions for its NF-κB-activating function (Kataoka and Tschopp, 2004). In light of reports showing that RIP binds MEKK3 and RIP over-expression activates MEKK3-dependent NF-κB activity (Blonska et al., 2004; Yang et al., 2001), it is tempting to speculate that TRAF proteins activate MEKK2, triggering NF-κB. Further, we propose that MC159–TRAF2 interactions are responsible for preventing MEKK2–IKK complex formation to prevent IκBβ degradation. An illustration of this proposed model is shown in Fig. 7. Alternatively, MC159 may associate directly with IκBβ/NF-κB complexes, similar to the cellular κB-ras molecule (Chen et al., 2003) to specifically prevent IKK-mediated IκBβ phosphorylation.

The homologous KSHV vFLIP induces IκBα phosphorylation and degradation (Sun et al., 2003) by binding to the IKK-gamma subunit, thereby activating the IKK complex (Field et al., 2003). Since MC159–IKK interactions were not detected previously (Chaudhary et al., 1999), it is unlikely that MC159 binds directly to IKK to induce selective degradation of IκBβ. It is also unlikely that MC159 binds to and inhibits specific NF-κB dimer populations since there was no detectable difference in the type of NF-κB subunits present in nuclei from vector- and MC159-expressing cells treated with TNF (Fig. 2B).

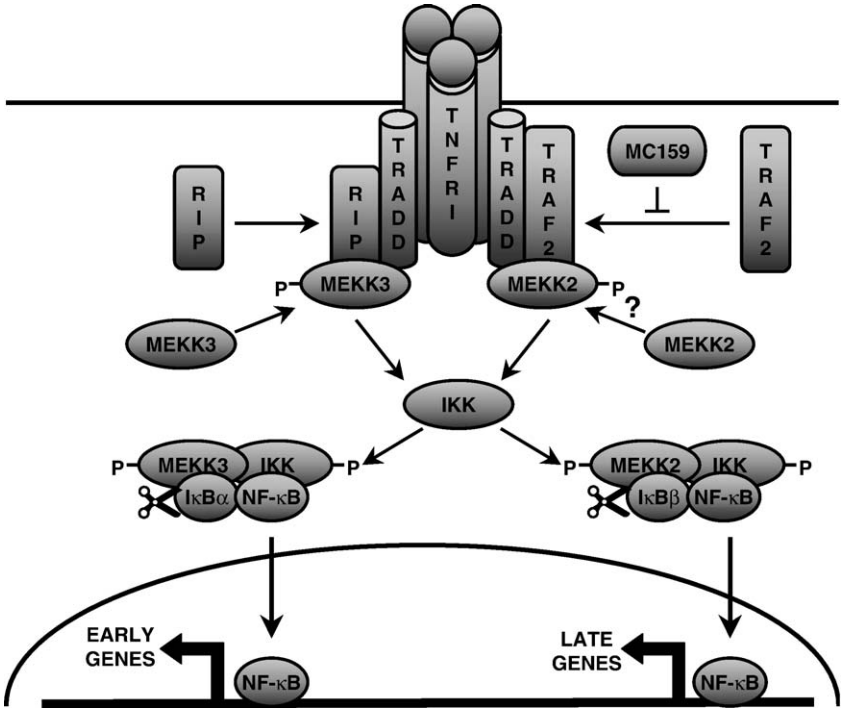

Fig. 7. A model of MC159 inhibition of the TNF-RI-mediated NF-κB activation pathway. TNF-RI activated by TNF-α recruits the adapter proteins TRADD, RIP and TRAF2. RIP activates MEKK3 to phosphorylate the IKK complex and activate the early phase of TNF-RI-induced NF-κB activation (left side of figure). A similar mechanism is proposed for TRAF2 and MEKK2 (right side of figure). MEKK3–IKK complexes in turn phosphorylate IκBα/NF-κB complexes, resulting to IκBα degradation and nuclear translocation of NF-κB, activating the early, but transient, phase of NF-κB activation. MEKK2–IKK complexes, in contrast, phosphorylate IκBβ:NF-κB complexes, resulting to IκBβ degradation and nuclear translocation of NF-κB, activating delayed, but persistent phase of NF-κB activation. Our current model is that MC159 proteins bind TRAF2 to specifically inhibit the IκBβ-mediated persistent activation of NF-κB.

Additionally, CXCL1, TNF and CCL-2 genes all require the same NF-κB dimers for expression (Hoffmann et al., 2003) but are affected differentially by the MC159 protein (Fig. 4). The effect of MC159 expression on other cellular genes expressed early or late after TNF-α treatment will also give further insight into MC159's molecular mechanism.

These studies were performed in the human 293T cell line. Although these cells are not the natural host for an MCV infection, we purposefully chose to use this cell line as a model system because it expresses high TNF-RI and low TNF-RII molecules. Future studies determining the effect of MC159 expression on TNF-α-mediated signaling in either transformed or primary keratinocytes are planned. Keratinocytes are the exclusive host cells for MCV infection. These cells produce TNF-α, a cytokine that induces macrophage migration, in response to a wide variety of stimuli. MCV-infected lesions are devoid of immune cells (Viac and Chardonnet, 1990) suggesting that chemoattractant molecules are absent at MCV infection sites. We show here that MC159 reduces the transcription of the CCL-2 gene, a monocyte chemoattractant protein, and TNF-α, which induces macrophage migration to sites of infection. MC159 protein expression may selectively inhibit the TNF-α-mediated production of specific chemoattractive molecules, resulting in localized immunosuppression and the characteristic benign MCV lesions. Future studies that detect mRNA and protein levels of these and other chemoattractant molecules in MC159-expressing cells over time will give great insight into the importance of MC159 expression during an MCV infection.

Materials and methods

Reagents

For all experiments, human embryonic kidney 293T cells (ATCC) were transiently transfected with the Fugene 6 transfection reagent (Roche). Recombinant human TNF-α (Roche) was used to activate NF-κB. Plasmids MC159/pCI and pCI and anti-MC159 antibody have been described previously (Shisler and Moss, 2001).

Luciferase assays

Luciferase assays were carried out as described previously (Shisler and Jin, 2004). 293T cells were co-transfected with 450 ng pNF-κB*luc* (Stratagene), 50 ng pRL-null (Promega) and either plasmid MC159/pCI or pCI. The pMC159A and pMC159B plasmids were a gift from Dr. Jeff Cohen (National Institutes of Health, Bethesda, MD) and have been described previously (Garvey et al., 2002a). pNF-κB*luc* contains the firefly luciferase gene under the control of an NF-κB-specific promoter. pRL-null expresses constitutively low levels of the sea pansy luciferase and is used to define transfection efficiencies. At 24 h post-transfection, cells were incubated with media containing TNF-α at the concentrations and times indicated in the figure legends. In some assays, cells were transfected with the pRIP plasmid (a gift from Dr. Preet Chaudhary, University of Texas Southwestern Medical Center). For these experiments, RIP over-expression was used instead of TNF-α to induce NF-κB activation. Cells were lysed and firefly and sea pansy luciferase activities of whole cell extracts were assayed by using the Dual Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate and firefly luciferase activity was divided by the sea pansy luciferase activity for each experimental point to normalize transfection efficiencies. The resultant ratios were normalized to that of untreated, pCI-transfected cells, whose value was taken as one.

Immunoblotting

Immunoblotting for cytoplasmic IκB has been previously described (Shisler and Jin, 2004). 293T cells were transfected with 1 μg of either plasmid MC159/pCI or pCI. At 24 h post-transfection, cells were incubated in media alone or media containing TNF-α (10 ng/ml). At varying times after TNF-α treatment, cells were harvested, collected by centrifugation and lysed in CE buffer at 4 °C for 5 min. Lysates were centrifuged briefly and supernatants containing cytoplasmic extracted proteins were assayed for protein concentration using the BCA Protein Assay Kit (Pierce). Equal amount of protein from each sample (25 μg) was resolved by 12% SDS-PAGE and transferred to a PVDF membrane. Blots were incubated with the indicated primary antibody and then incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce). Blots were developed using the Supersignal West Pico system (Pierce). The anti-IκBα and anti-IκBβ antibodies were purchased from Santa Cruz Biotechnology, and anti-actin antibody was purchased from Sigma.

In some experiments, cells were co-transfected with plasmid pTRAF2 (Dr. Carl Ware, La Jolla Institute for Allergy and Immunology) and either MC159/pCI, pMC159A or pMC159B. At 24 h post-transfection, cells were collected and lysates were incubated with Protein A-Sepharose beads (Amersham) and either anti-TRAF2 antisera (Santa Cruz Biotechnology) or rabbit IgG for 1 h at 4 °C while rocking. Beads were collected by centrifugation, washed and analyzed for the presence of MC159 by immunoblotting.

Electromobility shift assay (EMSA)

293T cells were transfected with one μg of either plasmid MC159/pCI or pCI. At 24 h post-transfection, cells were incubated in media absent for or containing TNF-α (10 ng/ml). At the times indicated, cells were harvested and collected by centrifugation, and nuclear proteins were extracted as

described previously (Oie and Pickup, 2001). Protein concentrations of nuclear extracted samples were quantified using the BCA Protein Assay Kit. Two μg of each extract was incubated with 1.75 pmol of ^{32}P-labeled NF-κB double stranded oligonucleotide (5′-AGTTGAGGGGACTTTCC-CAGGC-3′)(Promega) in 1X binding buffer as instructed in the Gel Shift Assay System (Promega). Reactions were resolved on a 6% acrylamide DNA retardation gel (Invitrogen) under non-denaturing conditions. The gel was dried on filter paper and exposed to a phosphorimager plate. For some reactions, nuclear extracted proteins were incubated with either anti-p65, anti-p50, anti-p52 or anti-cRel antisera (Santa Cruz Biotechnology) for 10 min at room temperature before the addition of radiolabeled probe to each reaction.

Reverse transcriptase PCR

293T cells were transfected with 1 μg of either plasmid MC159/pCI or empty vector. At 24 h post-transfection, cells were incubated with either media alone or media containing TNF-α (10 ng/ml). Cells were harvested and lysed after 0, 1 and 8 h. RNA was harvested using the RNeasy kit (Qiagen). Two μg of total RNA was incubated with 0.5 μg oligo(dT)$_{15}$ primer (Promega) and reverse transcribed at 42 °C using ImProm-II reverse transcriptase (Promega). One μl of the reverse transcription product was PCR amplified by incubating cDNA with 0.2 μM primers specific for either the CXCL1 (5′-ATGGCCCGCGCTG-CTCTCTCC-3′ and 5′-GTTGGATTTGTCACTGTTCAG-3′), CCL-2 (5′-GCCTCCAGCATGAAAGTCTC-3′ and 5′-GCTGCAGATTCTTTGGTTGT-3′) or TNF-α (5′-GAGT-GACAAGCCTGTAGCCCATGTTGTAGCA-3′ and 5′-GGCAATGATGATCCCAAAGTAGACCTGCCCAGACT-3′) genes. The human GAPDH gene was co-amplified in all PCR reactions, using primers (0.02 μM) specific for the GAPDH gene (5′-TGAAGGTCGGAGTCAACGGAT-TTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′). PCR reactions were analyzed by using agarose gel electrophoresis and amplicons were detected by ethidium bromide staining.

Acknowledgments

This research was supported by a grant to J.L.S. (AI055530) from the National Institutes of Health, and by start up funds provided by the School of Molecular and Cellular Biology at the University of Illinois, Urbana-Champaign. We thank Brian Nichols for technical support and Richard Tapping for critical review of the manuscript.

References

Bertin, J., Armstrong, R.C., Ottilie, S., Martin, D.A., Wang, Y., Banks, S., Wang, G.H., Senkevich, T.G., Alnemri, E.S., Moss, B., Lenardo, M.J., Tomaselli, K.J., Cohen, J.I., 1997. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc. Natl. Acad. Sci. U.S.A. 94 (4), 1172–1176.

Blonska, M., You, Y., Geleziunas, R., Lin, X., 2004. Restoration of NF-kappaB activation by tumor necrosis factor alpha receptor complex-targeted MEKK3 in receptor-interacting protein-deficient cells. Mol. Cell. Biol. 24 (24), 10757–10765.

Chan, F.K., Shisler, J., Bixby, J.G., Felices, M., Zheng, L., Appel, M., Orenstein, J., Moss, B., Lenardo, M.J., 2003. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J. Biol. Chem. 278 (51), 51613–51621.

Chaudhary, P.M., Jasmin, A., Eby, M.T., Hood, L., 1999. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene 18 (42), 5738–5746.

Chen, G., Goeddel, D.V., 2002. TNF-R1 signaling: a beautiful pathway. Science 296 (5573), 1634–1635.

Chen, Y., Wu, J., Ghosh, G., 2003. kappa B-Ras binds to the unique insert within the ankyrin repeat domain of Ikappa Bbeta and regulates cytoplasmic retention of Ikappa Bbeta /NF-kappa B complexes. J. Biol. Chem. 278 (25), 23101–23106.

Field, N., Low, W., Daniels, M., Howell, S., Daviet, L., Boshoff, C., Collins, M., 2003. KSHV vFLIP binds to IKK-gamma to activate IKK. J. Cell Sci. 116 (Pt. 18), 3721–3728.

Garvey, T., Bertin, J., Siegel, R., Lenardo, M., Cohen, J., 2002a. The death effector domains (DEDs) of the molluscum contagiosum virus MC159 v-FLIP protein are not functionally interchangeable with each other or with the DEDs of caspase-8. Virology 300 (2), 217–225.

Garvey, T.L., Bertin, J., Siegel, R.M., Wang, G.H., Lenardo, M.J., Cohen, J.I., 2002b. Binding of FADD and caspase-8 to molluscum contagiosum virus MC159 v-FLIP is not sufficient for its antiapoptotic function. J. Virol. 76 (2), 697–706.

Gil, J., Rullas, J., Alcami, J., Esteban, M., 2001. MC159L protein from the poxvirus molluscum contagiosum virus inhibits NF-kappaB activation and apoptosis induced by PKR. J. Gen. Virol. 82 (Pt. 12), 3027–3034.

Hiscott, J., Kwon, H., Genin, P., 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. J. Clin. Invest. 107 (2), 143–151.

Hoffmann, A., Levchenko, A., Scott, M.L., Baltimore, D., 2002. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science 298 (5596), 1241–1245.

Hoffmann, A., Leung, T.H., Baltimore, D., 2003. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J. 22 (20), 5530–5539.

Kataoka, T., Tschopp, J., 2004. N-terminal fragment of c-FLIP (L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. Mol. Cell. Biol. 24 (7), 2627–2636.

Oie, K.L., Pickup, D.J., 2001. Cowpox virus and other members of the orthopoxvirus genus interfere with the regulation of NF-kappaB activation. Virology 288 (1), 175–187.

Schmidt, C., Peng, B., Li, Z., Sclabas, G.M., Fujioka, S., Niu, J., Schmidt-Supprian, M., Evans, D.B., Abbruzzese, J.L., Chiao, P.J., 2003. Mechanisms of proinflammatory cytokine-induced biphasic NF-kappaB activation. Mol. Cell 12 (5), 1287–1300.

Seet, B.T., Johnston, J.B., Brunetti, C.R., Barrett, J.W., Everett, H., Cameron, C., Sypula, J., Nazarian, S.H., Lucas, A., McFadden, G., 2003. Poxviruses and immune evasion. Annu. Rev. Immunol. 21, 377–423.

Senkevich, T.G., Bugert, J.J., Sisler, J.R., Koonin, E.V., Darai, G., Moss, B., 1996. Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. Science 273 (5276), 813–816.

Senkevich, T.G., Koonin, E.V., Bugert, J.J., Darai, G., Moss, B., 1997. The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. Virology 233 (1), 19–42.

Shisler, J.L., Jin, X.L., 2004. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. J. Virol. 78 (7), 3553–3560.

Shisler, J.L., Moss, B., 2001. Molluscum contagiosum virus inhibitors of

apoptosis: The MC159 v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. Virology 282 (1), 14–25.

Sun, Q., Matta, H., Chaudhary, P.M., 2003. The human herpes virus 8-encoded viral FLICE inhibitory protein protects against growth factor withdrawal-induced apoptosis via NF-kappa B activation. Blood 101 (5), 1956–1961.

Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H., Tempst, P., Ghosh, S., 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. Cell 80 (4), 573–582.

Viac, J., Chardonnet, Y., 1990. Immunocompetent cells and epithelial cell modifications in molluscum contagiosum. J. Cutan Pathol. 17 (4), 202–205.

Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., Su, B., 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. Nat. Immunol. 2 (7), 620–624.

Zhou, A., Scoggin, S., Gaynor, R.B., Williams, N.S., 2003. Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference. Oncogene 22 (13), 2054–2064.