Host and parasite-derived IKK activities direct distinct temporal phases of NF-κB activation and target gene expression following *Toxoplasma gondii* infection

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Summary

Activation of NF-κB by the intracellular pathogen *Toxoplasma gondii* is associated with the localization of phosphorylated IκBα to the parasitophorous vacuole membrane (PVM). This is mediated by a parasite-derived IκB kinase (TgIKK) activity and is independent of host IKK function. In the present study, we examined the roles of host IKK and parasite-derived TgIKK on the temporal modulation of NF-κB activation. Despite the presence of TgIKK activity at the PVM, nuclear translocation of NF-κB and subsequent gene expression exhibited a requirement for the host IKK complex. A detailed kinetic analysis of NF-κB activation revealed a biphasic, hierarchical and temporally regulated response. We propose a novel paradigm for the modulation of NF-κB-dependent gene expression by *T. gondii* that involves both the host IKK complex and TgIKK activity at different phases of infection. Thus, *T. gondii* effectively alters gene expression in a temporal dimension by exploiting the NF-κB signaling machinery and subsequently rewiring the activation circuits of the infected host cell.

Supplementary material available online at
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Introduction

The NF-κB/Rel family of transcription factors is composed of five members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel. Each member of this family participates in the formation of homo- or heterodimers that are retained in the cytoplasm of resting cells by inhibitor-kappa B (IκB) proteins (Ghosh and Karin, 2002). Nuclear translocation of NF-κB dimers is dependent on the phosphorylation, ubiquitination and degradation of IκB proteins, primarily IκBα, by the IκB kinase (IKK) complex following cell stimulation (Hayden and Ghosh, 2004; Mercurio et al., 2000). Once in the nucleus, NF-κB regulates the transcription of several genes involved in immune responses, cell proliferation, survival and apoptosis (Ghosh and Karin, 2002; Karin and Lin, 2002; Richmond, 2002).

It is well recognized that microbial pathogens have evolved diverse strategies to manipulate the NF-κB pathway (Tato and Hunter, 2002). Inhibition of NF-κB activation has the potential of interfering with the development of antimicrobial immune responses, thus providing a survival advantage to the infectious agent. Additionally, activation of NF-κB might upregulate expression of anti-apoptotic genes and prevent death of infected cells in order to allow replication of the pathogen. The obligate intracellular protozoan *Toxoplasma gondii* has recently become a model organism for the study of mechanisms involved in the subversion of NF-κB and apoptotic pathways (Denkers et al., 2004; Mason et al., 2004a; Sinai et al., 2004). The rapidly growing stage of the parasite known as the tachyzoite develops within a parasitophorous vacuole (PV) in the infected cell (Tenter et al., 2000). Studies from our laboratory have shown that phosphorylated IκBα localizes to the PV membrane (PVM) surrounding the PV in *T. gondii*-infected cells (Molestina et al., 2003). In addition, infected cells exhibit nuclear translocation of p50 and p65 (RelA), which correlates with the induction of anti-apoptotic genes, such as members of the Bcl-2 family and inhibitor of apoptosis proteins (IAP) (Molestina et al., 2003). These events probably underlie the mechanisms involved in the resistance of *T. gondii*-infected cells to apoptotic stimuli. Accordingly, infection of p65$^{-/-}$ (RelA$^{-/-}$) mouse embryonic fibroblasts (MEFs) results in a loss of the anti-apoptotic phenotype and a decrease in pro-survival gene expression (Molestina et al., 2003; Payne et al., 2003).

The IKK complex comprises two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (NEMO) (Ghosh and Karin, 2002; Hayden and Ghosh, 2004). Gene-knockout and enzymological studies have defined IKKβ as the main catalytic subunit of the IKK complex, possessing the bulk of the IκBα phosphorylation activity at serine residues 32 and 36 (Cao et al., 2001; Li et al., 1999; Li et al., 2000; Wisniewski et al., 1999). Surprisingly, the accumulation of phospho-IκBα at the PVM in *T. gondii*-infected cells is not caused by recruitment and activation of either IKKβ or IKKα but by a unique parasite-derived activity designated TgIKK that exhibits a similar specificity for Ser32 and Ser36 as the mammalian IKK complex (Molestina and Sinai, 2005).

We reasoned that the phosphorylation of IκBα at the PVM

by TgIKK might serve as the mediator of NF-κB activation promoting both nuclear translocation of NF-κB subunits and the accompanying upregulation of gene expression. In the present study, we address the roles of both the host IKK and parasite-derived TgIKK activities in the *T. gondii*-dependent activation of NF-κB target genes. Our results reveal a complex regulatory pattern of NF-κB activation that is crucially dependent on the integrity of the host IKK complex early in infection but requires TgIKK activity to sustain increased levels of gene expression. This response appears to be tailored by the parasite to attain selective modulation of host genes, which include crucial cytokines and pro-survival factors.

Materials and Methods

Cell lines and parasite

Immortalized wild-type (WT) MEFs and Vero cells (ATCC CCL-81) were maintained in alpha minimum essential medium supplemented with 7% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Gibco BRL). IKKα$^{-/-}$ and IKKβ$^{-/-}$ MEFs were provided by M. Karin (University of California San Diego, San Diego, CA). IKKα$^{-/-}$β$^{-/-}$ MEFs were a gift from I. Verma (Salk Institute for Biological Studies, San Diego, CA). The RH strain of *T. gondii* with a deletion in the gene encoding hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT; NIH AIDS Research and Reference Reagent Program) (Donald et al., 1996) was used exclusively and maintained by serial passage in Vero cells as previously described (Sinai et al., 2000).

Immunofluorescence

Confluent WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs were grown on sterile 12 mm glass coverslips placed in 24-well plates and infected at a multiplicity of infection (m.o.i.) of 5:1 with freshly passaged parasites for different periods of time. For short-term incubations (1-9 hours), the inoculum was centrifuged onto the cell monolayer at 800 *g* for 5 minutes at 4°C to synchronize invasion of MEFs. Procedures for immunofluorescence analysis (IFA) were performed as described previously (Molestina et al., 2003). Primary antibodies used in these experiments were: anti-phospho-IκBα Ser32 antibody (Santa Cruz Biologicals, cat. no. sc8404), anti-p50 antibody (provided by N. Rice, National Cancer Institute, Bethesda, MD), anti-p65 antibody (Santa Cruz Biologicals, cat. no. sc-372), anti-GRA3 antibody (Bermudes et al., 1994), and anti-SAG1 antibody (Argene). Species-specific Oregon-Green- or Texas-Red-conjugated secondary antibodies were purchased from Molecular Probes.

Immunoblot analysis

WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs were seeded separately in 6-well plates at 2×10^5 cells/well and allowed to adhere overnight. Cells were infected with freshly passaged parasites at an m.o.i. of 5:1 for the time points indicated. For short-term incubations (1-9 hours), the inoculum was centrifuged onto the monolayer at 800 *g* for 5 minutes at 4°C to synchronize invasion of cells. Immunoblot analysis of IκBα phosphorylation (Ser32) in whole-cell lysates and p50/p65 translocation in nuclear extracts was performed as described previously (Molestina et al., 2003). Equal amounts of protein (20 µg for IκBα phosphorylation and 10 µg for nuclear translocation assays) were resolved for each cell line by SDS-PAGE prior to immunoblotting, and signals were detected using a chemiluminescence-based system (Pierce). Identical exposures, based on the signal in wild-type cells, were used on all samples. Where indicated, densitometric analysis of protein bands was performed using the ImageJ software (http://rsb.info.nih.gov/ij/). Integrated

densitometric values (IDV) of infected cells were corrected for IDVs of protein bands from uninfected cells (0 hour time point).

Electrophoretic mobility shift assay

Extraction of nuclear proteins from infected cells and binding reactions for electrophoretic mobility shift assays (EMSA) were performed as reported elsewhere (Molestina et al., 2000; Molestina et al., 2003). Supershift assays were performed with antibodies to p50 and p65.

Real-time RT-PCR analysis

Primers and reagents for real-time RT-PCR were purchased from SuperArray. RNA was isolated from uninfected and infected WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ cells after 0, 1, 3, 6, 9 and 24 hours of incubation using RNeasy Mini kits (Qiagen). Synthesis of cDNA was performed with 1 µg of total RNA in a 25 µl reaction mixture containing 1×RT reaction buffer, 1 µg random hexamers, 0.5 mM dNTPs, 1 U RNAse inhibitor, and 10 U MMLV reverse transcriptase at 37°C for 1 hour. Real-time PCR assays included the addition of HotStart Taq DNA polymerase in a 25 µl reaction mixture containing 1 µl cDNA, 10 mM Tris-Cl, 50 mM KCl, 2 mM MgCl$_2$, 0.2 mM dNTPs, 1 µl gene-specific primer mix and 1× SYBR Green I solution (Molecular Probes). Reactions were run at 95°C for 15 minutes and 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. A LightCycler 2.0 Instrument (Roche Diagnostics) was used to detect and record fluorescence signals from each reaction. Threshold cycle (Ct) values were calculated for a particular gene at the different time points examined using the instrument software. Differences in the levels of gene expression over time were determined for each condition line by relative quantification using the Delta Delta Ct (ΔΔCt) method as suggested by the manufacturer (SuperArray). The numerical data were subjected to analysis of variance followed by the Bonferroni post-test using the GraphPad Prism software. A *P* value of <0.05 was used to determine statistical significance.

NF-κB gene arrays

Unless stated otherwise, all reagents used in gene array experiments including specific primer sets and hybridization membranes were purchased from SuperArray (mouse NFκB array, cat. no. MM-016). Infection of WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs with *T. gondii* was performed at a m.o.i. of 5:1 for 24 hours. Total RNA from both uninfected and infected cells was isolated and 5 µg used as template for synthesis of [α-^{32}P]dCTP-labeled (ICN) cDNA probes with an NF-κB array-specific primer set. Hybridization conditions of cDNA probes and calculation of IDVs from cDNA signals hybridized to each gene were performed as described (Molestina et al., 2003). To determine fold differences in gene expression after infection, IDVs were normalized to actin since glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known to be upregulated by *T. gondii* infection (Blader et al., 2001). Intensity ratios between infected and uninfected cells were calculated for each gene using the GEArray Analyzer software (SuperArray).

Results

TgIKK activity at the PVM correlates with parasite replication

We determined the kinetics of the appearance of phospho-IκBα at the PVM as an indicator of TgIKK activity during infection. Following parasite invasion, PVs were examined for the presence of phospho-IκBα at early (1-3 hours), intermediate (6-9 hours) and late periods of infection (18-24 hours) by IFA.
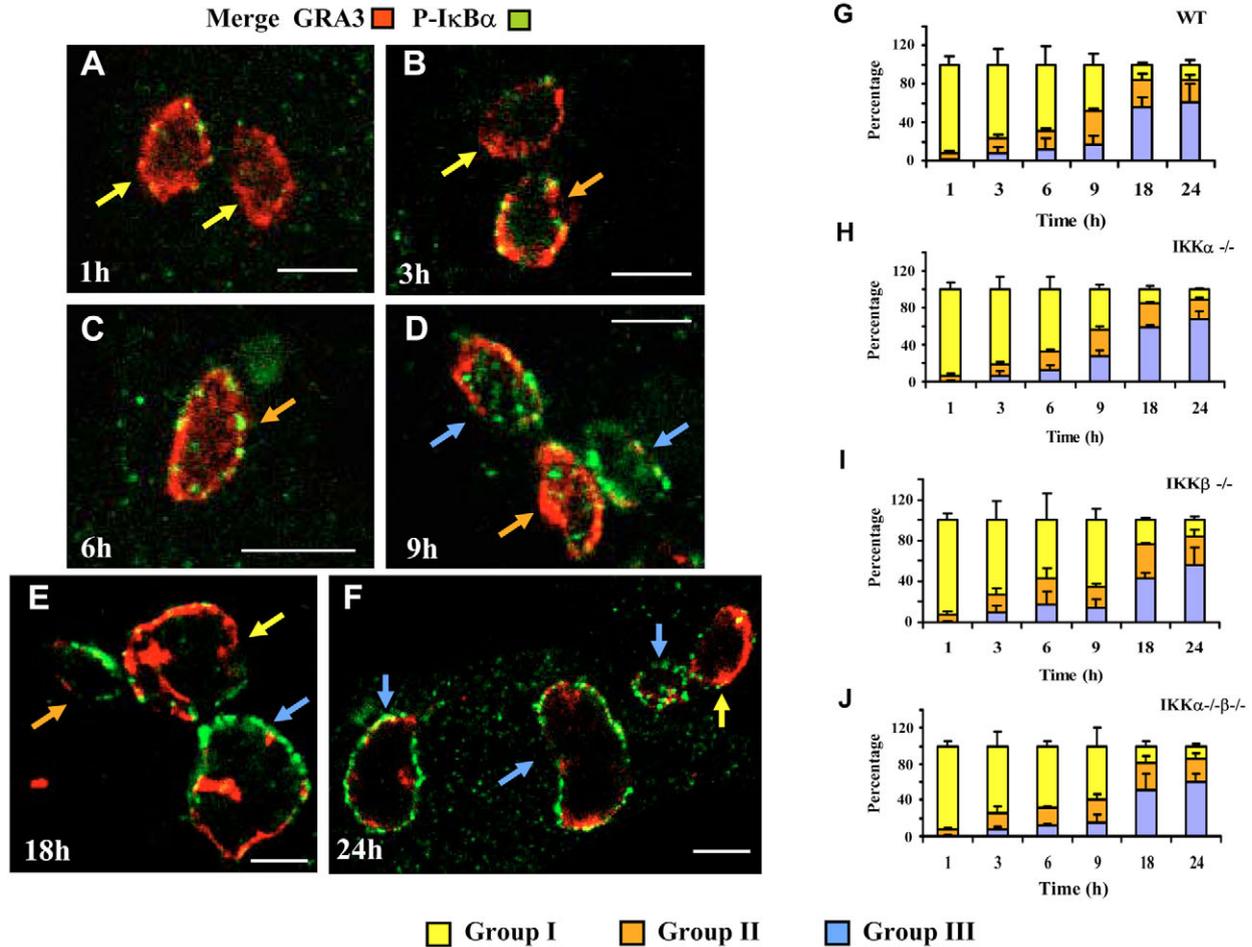
Fig. 1. Kinetics of phospho-IκBα localization at the *T. gondii* PVM as an indicator of TgIKK activity. (A-F) IKKα$^{-/-}$β$^{-/-}$ MEFs were cultured on glass coverslips and infected with freshly passaged *T. gondii* tachyzoites at an m.o.i. of 5:1 for the times indicated. Double immunofluorescence was performed with mouse monoclonal anti-phospho-IκBα Ser32 antibody (P-IκBα; green) and rabbit polyclonal anti-*T. gondii* GRA3 antibody (red). Different levels of phospho-IκBα coverage at the PVM allowed the classification of vacuoles into three groups: group I showed 0-25% of the PVM covered with phospho-IκBα (yellow arrows), group II displayed 50-75% coverage (orange arrows), and group III showed 75-100% of their surface covered with phospho-IκBα (blue arrows). Bars, 6 μm. (G-J) Quantification of the different populations of vacuoles indicated a time-dependent increase in phospho-IκBα localization at the PVM. WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs were cultured on glass coverslips and infected with *T. gondii* at an m.o.i. of 5:1 for the times indicated. A minimum of 300 vacuoles were counted under 100× magnification in a blinded fashion for each cell line and time point. Data represent means ± s.d. of three separate experiments individually quantified by both authors.

Since the volume of the *T. gondii* vacuole increases with parasite replication, we used the proportion of the PVM surface covered by phospho-IκBα as a semiquantitative measure of PVM-localized TgIKK activity. Accordingly, we defined three groups of vacuoles (Fig. 1): group I included vacuoles with 0-25% of the PVM decorated with phospho-IκBα (Fig. 1A, yellow arrows); group II vacuoles featured 25-75% coverage (Fig. 1B-E, orange arrows); and group III vacuoles displayed 75-100% of their surface covered with phospho-IκBα (Fig. 1D-F, blue arrows). Images in Fig. 1 were obtained from infected IKKα$^{-/-}$β$^{-/-}$ MEFs, validating the exclusive role of the parasite TgIKK in the PVM localization of phospho-IκBα. WT, IKKα$^{-/-}$ and IKKβ$^{-/-}$ MEFs displayed equivalent staining patterns of phospho-IκBα at the PVM throughout infection as described below.

We quantified the proportions of each group of vacuoles in infected WT and IKK-knockout cells as a measure of TgIKK

activity at the PVM as a function of time of infection. As shown in Fig. 1G-J, the early phase of infection (1-3 hours) was characterized predominantly by group I vacuoles, indicating little-to-no PVM-associated phospho-IκBα following invasion. The intermediate phase of infection, which correlates with the completion of the first round of parasite replication (6-9 hours), showed a noticeable increase in localized phospho-IκBα coincident with a rise in group II and III vacuoles. The highest levels of phospho-IκBα at the PVM were seen at the late phase of infection (18-24 hours), featuring a high proportion of group III vacuoles (Fig. 1G-J). A small proportion of vacuoles with a low level of staining were still observed at this late phase as depicted by the yellow arrows in Fig. 1E,F. The increase in phospho-IκBα localization observed over time was not restricted to WT MEFs since essentially identical kinetic profiles were observed in IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs (Fig. 1G-J). Therefore, the appearance of phospo-IκBα

at the PVM does not correlate with parasite invasion but rather by parasite growth.

An intact IKK complex is crucial for activation of NF-κB early in infection

The lack of TgIKK activity early in infection suggests that the host IKK might play an instrumental role in activation of NF-κB in the early phase of infection. To determine whether the kinetics of IκBα phosphorylation were affected by a loss of IKK function at the population level, analysis of phospho-IκBα was performed by immunoblotting. As shown in Fig. 2A, an increase in IκBα phosphorylation was apparent by 1 hour post-infection (p.i.) in WT MEFs at a phase where TgIKK activity was negligible (Fig. 1G). Interestingly, levels of IκBα phosphorylation were maximal at 9 hours p.i. (Fig. 2A) concurrent with an increase in TgIKK activity at the PVM (Fig. 1G). A slight decrease in IκBα phosphorylation was consistently observed at 24 hours p.i.; however, this response was still higher than uninfected cells, suggesting persistent activation (Fig. 2A, compare the time points for 0 and 24 hours). The kinetic profile of IκBα phosphorylation in IKKα$^{-/-}$ MEFs was essentially identical to WT MEFs (Fig. 2B). As reported previously (Molestina et al., 2003), a marked degradation of IκBα was not observed throughout infection despite elevated levels of phosphorylation (Fig. 2A,B).

Contrary to WT and IKKα$^{-/-}$ MEFs, only a slight elevation in IκBα phosphorylation was detected in IKKβ$^{-/-}$ MEFs (Fig. 2C), whereas infection of IKKα$^{-/-}$β$^{-/-}$ cells was characterized by a distinct absence of IκBα phosphorylation throughout infection (Fig. 2D). The failure to promote significant levels of
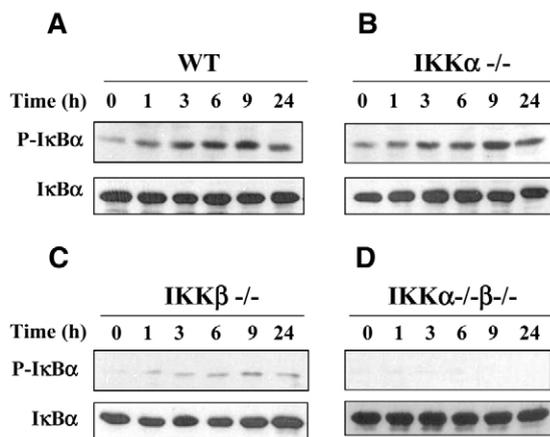

Fig. 2. Kinetic analysis of IκBα phosphorylation in total cell populations reveals a requirement for host IKK. WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs were infected at an m.o.i. of 5:1 for the times indicated. Immunoblots were performed with antibodies against phospho-IκBα (Ser32) and IκBα as described in the Materials and Methods. Elevated levels of phospho-IκBα (P-IκBα) were apparent by 1 hour p.i. in WT (A) and IKKα$^{-/-}$ (B) cells. Phosphorylation of IκBα was maximal at 9 hours p.i. (A,B), which is concurrent with the increase in TgIKK activity at the PVM observed in Fig. 1. Contrary to this, only a slight elevation in IκBα phosphorylation was detected in IKKβ$^{-/-}$ cells (C) and a lack of phosphorylation was observed in IKKα$^{-/-}$β$^{-/-}$ cells (D). The levels of IκBα in all cell lines examined were comparable between uninfected (0 hour time point) and *T. gondii*-infected cells.

phospho-IκBα in IKKβ and IKKα$^{-/-}$β$^{-/-}$ cells, even in the presence of increased TgIKK activity (Fig. 1I and J, 9-24 hours), suggests a localized signal deriving primarily from a subset of IκBα molecules at the PVM (Molestina and Sinai, 2005). In addition, the results implicate IKKβ as the major catalytic subunit acting on the substantially larger pool of non-PVM-associated IκBα.

Roles of host IKK and TgIKK in the *T. gondii*-mediated nuclear translocation of NF-κB

Phosphorylation of IκBα is the crucial event promoting nuclear translocation of NF-κB. In the absence of TgIKK activity at the PVM early in infection, we reasoned that translocation of NF-κB at this stage would indicate involvement of the host IKK complex. Likewise, a late response would correlate with an increase in TgIKK activity at the PVM at a time of host IKK activity dampening (Hoffman et al., 2002; Nelson et al., 2004).

The kinetics of p50 and p65 translocation in WT MEFs (Fig. 3A,B and C,D, respectively) indicated a profile that paralleled the phosphorylation of IκBα (Fig. 2A). The profile presents as a distinctly biphasic event with a second sustained 'wave' of NF-κB translocation correlating with the appearance of TgIKK at the PVM. Surprisingly, despite near-WT phosphorylation levels of phospho-IκBα, infection of IKKα$^{-/-}$ cells exhibited a profound defect in the nuclear translocation of both p50 (Fig. 3A,B) and p65 (Fig. 3C,D), particularly early in the infection. However, a small but reproducible increase in p50 and p65 levels in the nucleus was observed by 9 to 24 hours p.i., consistent with the appearance and accumulation of TgIKK at the PVM. Nuclear translocation of p50 and p65 was similarly deficient at early stages of infection in the IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ backgrounds but the IKKα$^{-/-}$β$^{-/-}$ cells failed to show a steady increase even at 18-24 hours p.i.

Analysis of NF-κB translocation in response to *T. gondii* infection was also examined by IFA (Fig. 4). Nuclear translocation of p50 (Fig. 4A) and p65 (Fig. 4B) was observed in WT, IKKα$^{-/-}$ and IKKβ$^{-/-}$ cells, but was never detected in IKKα$^{-/-}$β$^{-/-}$ cells as examined at 24 hours p.i., highlighting the requirement for an intact IKK signalosome. The capacity for NF-κB translocation exhibited a correlation with the reported catalytic activity of the IKK subunits (Cao et al., 2001; Li et al., 1999): the extent of the response, although indistinguishable between WT and IKKα$^{-/-}$ cells after 24 hours, displayed heterogeneity in infected IKKβ$^{-/-}$ cells. In these cells, a proportion of infected cells failed to show p65 translocation (Fig. 4B, yellow arrow). Of note, even infected WT cells did not display complete translocation of these subunits, as one would detect with a potent trigger such as tumor necrosis factor α (TNF-α; data not shown) (Molestina et al., 2003), because a considerable amount of cytoplasmic staining was observed.

We performed EMSAs to examine the effect of infection on NF-κB binding activity. As shown in Fig. 5A, incubation of a radiolabeled oligonucleotide probe harboring an NF-κB consensus sequence with nuclear extracts of uninfected (U) and infected (I) WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ cells resulted in distinct binding activities of three complexes (C1, C2 and C3). Infection resulted in a lower binding activity of the C1 complex in WT MEFs, whereas this response remained unaffected or increased in IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$

Fig. 3. A disruption in the integrity of the host IKK complex causes defective NF-κB translocation in response to infection. Nuclear extracts were prepared from WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and double-knockout IKKα$^{-/-}$β$^{-/-}$ MEFs after different periods of infection with *T. gondii.* Cells were infected at an m.o.i. of 5:1. Translocation of p50 (A) and p65 (C) was examined by immunoblotting, and densitometric analysis of corresponding protein bands (B,D) was performed as described in the Materials and Methods. The kinetics of p50 (A,B) and p65 (C,D) translocation in WT cells paralleled the robust response seen with the phosphorylation of IκBα shown in Fig. 2. Contrary to this, only moderate increases in p50 and p65 translocation were observed in all IKK mutant cell lines at early stages of infection. IDV, integrated densitometric value.
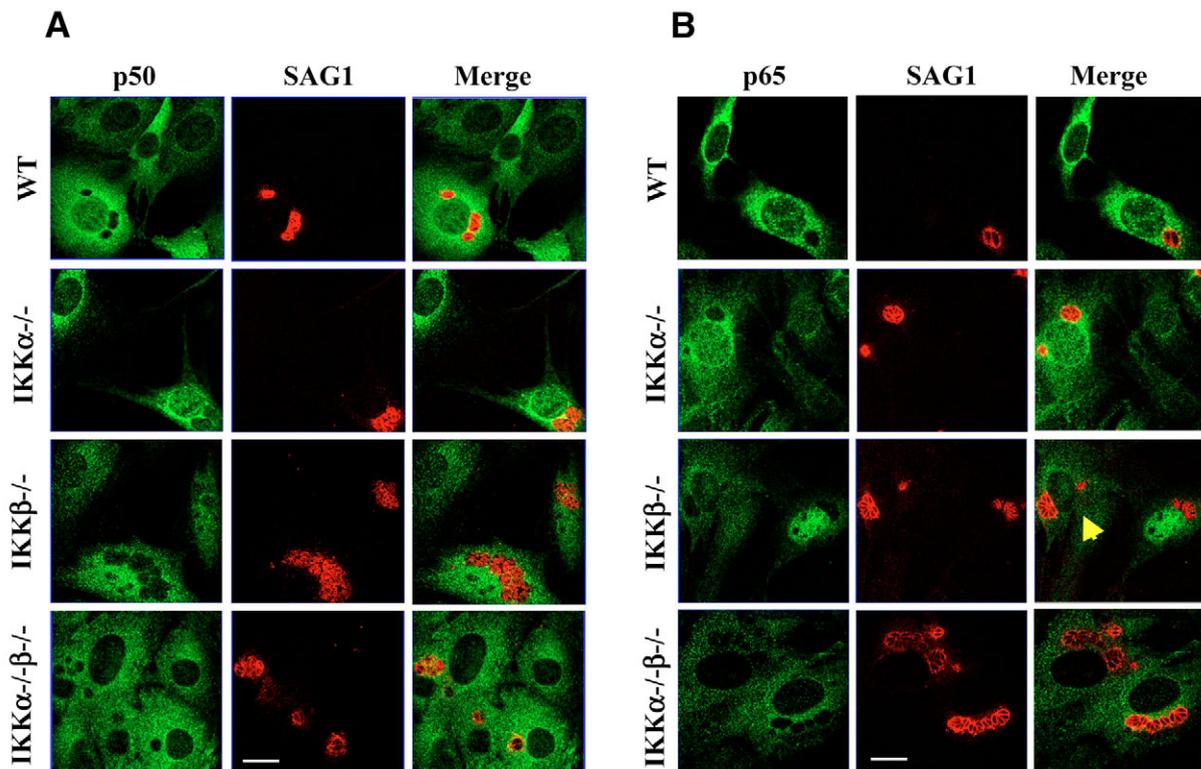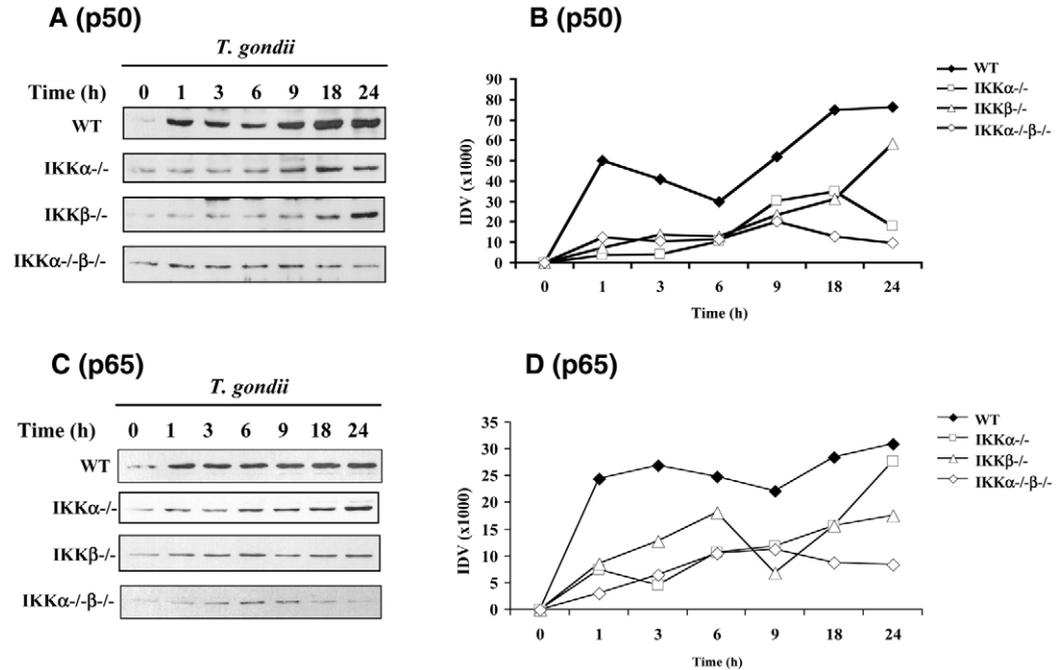

Fig. 4. IFA analysis of NF-κB localization in WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs infected with *T. gondii* for 24 hours. Double immunofluorescence labeling was performed with antibodies against p50 (A) and p65 (B), and anti-*T. gondii* SAG1. Translocation of p50 and p65 is observed in infected WT, IKKα$^{-/-}$ and IKKβ$^{-/-}$ cells but not in uninfected cells. A proportion of infected IKKβ$^{-/-}$ cells fails to show p65 translocation at a similar extent as WT and IKKα$^{-/-}$ cells (yellow arrow). Infected IKKα$^{-/-}$β$^{-/-}$ cells do not display nuclear localization of p50 or p65. Bars, 20 μm.

Fig. 5. *T. gondii* infection results in different levels of NF-κB DNA binding activity depending on the IKK background of the host cell. (A) Binding reactions for EMSA were performed with a radiolabeled oligonucleotide probe containing the NF-κB consensus sequence and nuclear protein extracts from uninfected (U) or infected (I) cells. Diverse NF-κB binding activities of three complexes (C1, C2 and C3) are observed among WT, IKKα^{–/–}, IKKβ^{–/–} and IKKα^{–/–}β^{–/–} cells with infection. Increases in binding activity of complexes C2 and C3 are observed in infected WT and, to a lesser extent, in IKKα^{–/–} cells compared with uninfected controls (dashed box). Levels of NF-κB binding activity of C2 and C3 complexes are minimal in infected IKKβ^{–/–} cells and absent in infected IKKα^{–/–}β^{–/–} cells. (B) Supershift analysis of infected WT, IKKα^{–/–} and IKKβ^{–/–} cells determined the presence of p50/p65 and p50/p50 dimers in the C2 and C3 complexes, respectively (arrowheads). The disappearance or marked reduction of C1 with the p50 but not the p65 antibody suggests the presence of p50 subunits in this complex, which might also consist of additional unidentified proteins.
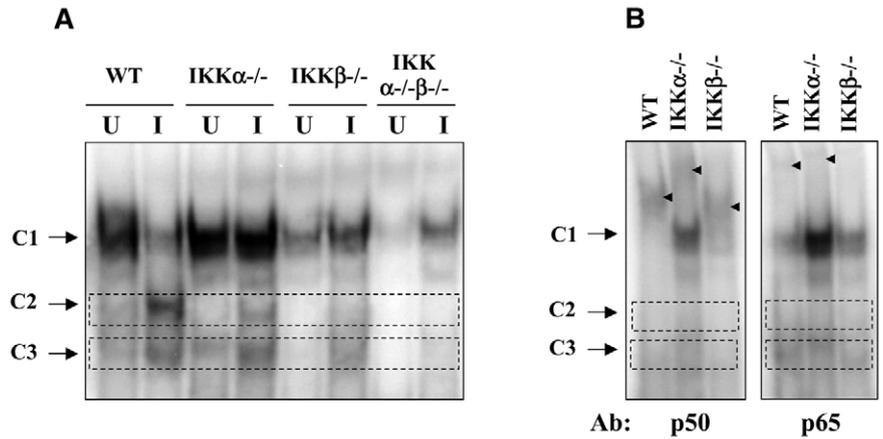
cells. Increased NF-κB binding activities were consistently observed with complexes C2 and C3 in infected WT cells and to a lesser extent in IKKα^{–/–} cells (Fig. 5A, dashed box). This response was markedly reduced in IKKβ^{–/–} cells and absent in IKK double-knockout cells. Supershift analysis of infected WT, IKKα^{–/–} and IKKβ^{–/–} cells determined the presence of p50/p65 and p50/p50 dimers in the C2 and C3 complexes, respectively (Fig. 5B, dashed box). The disappearance (WT and IKKβ^{–/–} cells) or marked reduction (IKKα^{–/–} cells) of C1 with p50 but not with p65 antibody suggests the presence of p50 subunits in this complex in addition to other potential proteins whose identities are yet to be determined.

T. gondii infection induces an IKK-subunit-dependent modulation of gene expression

The results presented above show that a disruption in any of the catalytic components of the host IKK complex results in a defective NF-κB translocation response to infection even in the presence of optimal levels of IκBα phosphorylation as seen with IKKα^{–/–} MEFs. We reasoned that the modulation of gene expression in the host cell might be controlled in a hierarchical fashion by sequential contributions from endogenous IKK and parasite-derived TgIKK activities. The steady state levels of gene expression were initially examined at 24 hours p.i. to provide an overview of the NF-κB-dependent transcriptional response induced by *T. gondii*.

DNA hybridization arrays focused on the NF-κB pathway were probed with radiolabeled cDNA from uninfected and *T. gondii*-infected cells. Arrays consisted of 65 genes implicated in the regulation of the NF-κB pathway and 31 NF-κB-responsive genes. The results in Fig. 6 represent fold changes in gene expression between infected and uninfected cells and depict the means of three experiments. The complete data sets with means ± s.d. representing changes in expression for each gene in response to infection are shown in Table S1 (supplementary material).

Infection of WT MEFs resulted in a robust upregulation of several genes coding for transmembrane receptors, adaptor molecules, members of the NF-κB family and targets of NF-κB (Fig. 6). Increases in gene expression of at least threefold were notably observed among members of the Toll-like receptor (TLR) and TNF-receptor-associated factor (TRAF) families, as well as regulatory kinases affecting IKK activity such as MEKK1 and TAK1. Within the NF-κB family, similar increases were observed among NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA, RelB and the IκBs. Approximately 85% of NF-κB target genes were found to be upregulated by at least twofold by *T. gondii* infection in WT cells (Fig. 6). These included several early response genes previously found to be induced by *Toxoplasma* infection in human foreskin fibroblasts (HFF) such as interleukin 6 (IL-6), IL-1β, intercellular cell adhesion molecule 1 (ICAM-1) and interferon regulatory factor 1 (IRF-1) (Blader et al., 2001).

The overall transcriptional response in IKK mutant cells was considerably reduced among key regulators of the NF-κB pathway and NF-κB target genes (Fig. 6). Although 50% of all genes in the array were upregulated by at least threefold in WT MEFs, only 12% in IKKα^{–/–}, 6% in IKKβ^{–/–} and 4% in IKKα^{–/–}β^{–/–} MEFs showed a similar response. Interestingly, nearly 30% of all genes in the array were downregulated by threefold or more in IKKα^{–/–}β^{–/–} cells as a result of infection. These included genes that were markedly upregulated in WT cells (Fig. 6). Such a downregulatory trend was not as dramatic among cells lacking individual IKK subunits. In fact, a tendency for subsets of genes showing different levels of expression dependent on either IKKα or IKKβ was observed. Thus, the induction of TRAF1, TRAF2, RelA and the ICAMs was affected to a higher extent by the lack of IKKα compared with IKKβ. Likewise, levels of TLR1, TLR2, IκBβ, RelB and TNF-α expression showed a dependency on IKKβ (Fig. 6). An exception to the broad suppression of gene expression in IKKα^{–/–}β^{–/–} MEFs was the induction of early growth response 1 (EGR1) and interferon α (IFN-α). Levels of expression of these genes were at least fourfold greater in all cell lines, reflecting the capability of *T. gondii* to trigger diverse pathways of activation independent of IKK (Blader et al., 2001).

Altogether, the divergence of gene expression profiles affected by the lack of one or two IKK subunits during
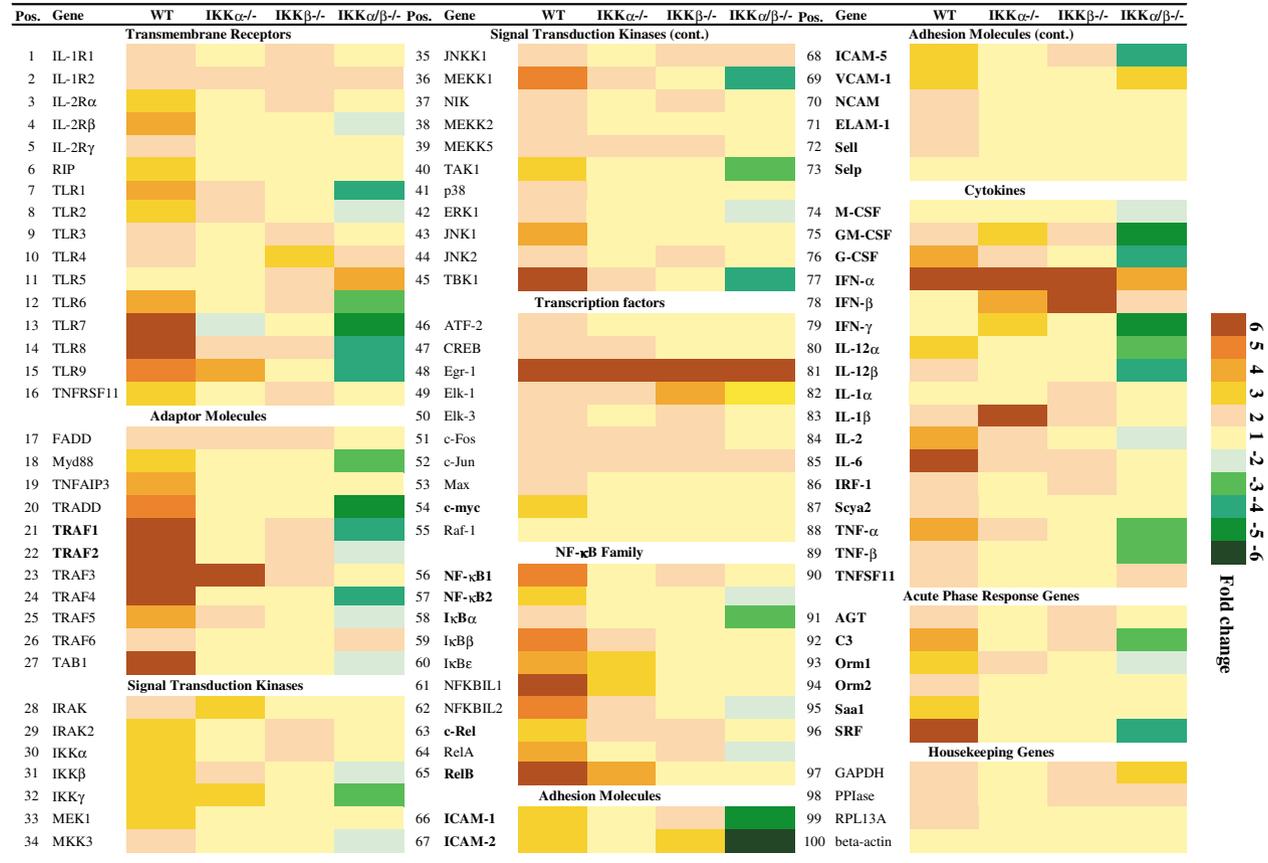
Fig. 6. Modulation of host cell genes involved in the regulation of the NF-κB pathway by *T. gondii*. Densitometric values were normalized to actin, and levels of gene expression are shown as infected/uninfected ratios (see color key showing relative fold change). Data represent the means of three experiments performed separately. The complete data sets with means ± s.d. are shown in Table S1 (supplementary material). Target genes of NF-κB are shown in bold. Pos. indicates position in the array.

infection supports an essential role for the integrity of an active complex in the host cell. In addition, these results suggest that the integration of signals deriving from both an active IKK signalosome early in infection and TgIKK late in infection affects the magnitude of the NF-κB-dependent transcriptional response.

Temporal analysis of gene expression reveals a biphasic response during infection

The array experiments revealed an induction of several early response genes, including pro-inflammatory cytokines whose upregulation was affected by the IKK background of the infected host cell. As representative genes from this group, we examined the kinetics of IL-6 and GRO1 expression by real-time quantitative RT-PCR. Infection of WT cells resulted in fluctuating patterns of induction of these genes (Fig. 7A,B, respectively). These profiles suggest distinct stages of activation that can be condensed into two main phases determined by an early host IKK response (1-3 hours) and a subsequent TgIKK-dependent component later in infection (9-24 hours). This biphasic response in gene expression was not clearly defined in the IKK mutant cell lines owing to the marked abrogation in IL-6 and GRO1 expression compared

with WT (Fig. 7A,B, respectively). More importantly, in the absence of the early IKK-dependent response, the appearance of TgIKK at the PVM at intermediate and late stages of infection (Fig. 1) is unable to promote high levels of activation of these genes.

To determine whether the biphasic response observed with IL-6 and GRO1 was attributable to early response genes only, we examined the temporal regulation of IAP2, which is an NF-κB-dependent anti-apoptotic gene previously reported to be induced late in infection (Blader et al., 2001; Molestina et al., 2003). As shown in Fig. 7C, the expression of IAP2 between early and late stages of infection was not substantially different compared with the characteristic biphasic response observed with IL-6 and GRO1 in WT MEFs. In addition, disruptions in one or both catalytic subunits of the IKK complex resulted in only moderate decreases in IAP2 expression as opposed to the marked reduction observed with IL-6 and GRO1 (Fig. 7A,B). These results reveal that the temporal patterns of expression of different NF-κB target genes are regulated selectively by the hierarchical relationship of host IKK and TgIKK activities. Moreover, additional levels of control downstream of IKK and TgIKK might govern which genes are activated and their magnitude of expression.
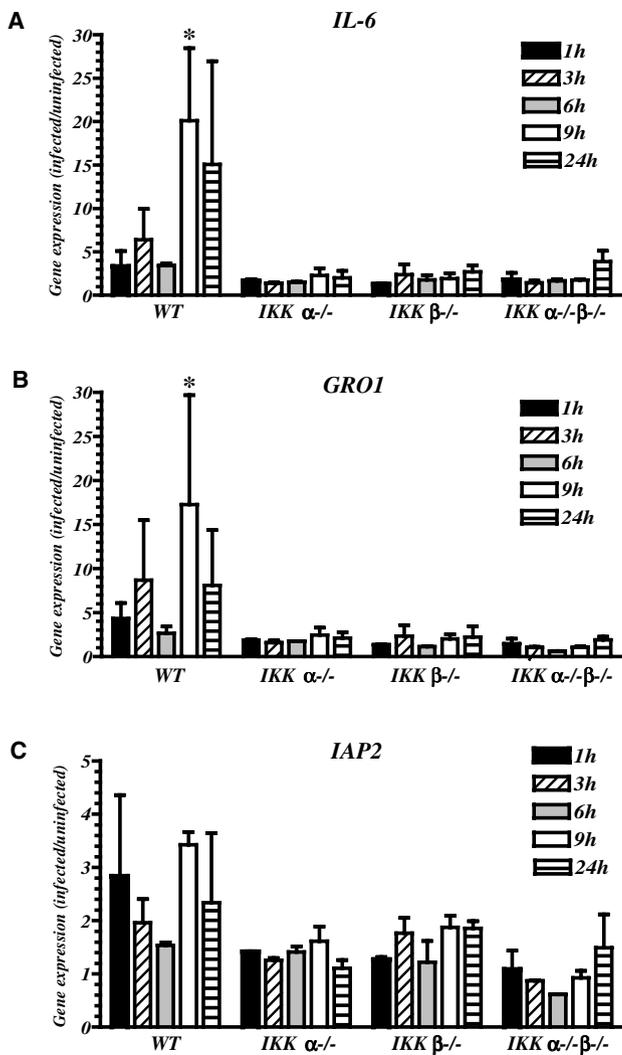
A

B

C

Fig. 7. Temporal analysis of gene expression by *T. gondii* infection. WT, IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ MEFs were infected with *T. gondii* at an m.o.i. of 5:1 and total RNA was isolated at the time points indicated. Expression of IL-6 (A), GRO1 (B) and IAP-2 (C) was determined by real-time RT-PCR analysis as described in the Materials and Methods. Infection of WT cells resulted in fluctuating patterns of IL-6 (A) and GRO1 (B) activation; these fell into two major phases determined by an early host IKK response (1-3 hours) and an intermediate-to-late TgIKK response (9-24 hours). Contrary to this, a marked decrease of IL-6 and GRO1 expression was observed in IKK-knockout cells. A disruption of the IKK complex did not abrogate the expression of IAP2 to the same extent as observed with IL-6 and GRO1 (C), thus the temporal analysis of IAP2 lacked the characteristic biphasic response observed with early response genes. *, $P<0.05$ when comparing the levels of expression at the 9 hours time point of WT cells to all other time points in IKKα$^{-/-}$, IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ cells.

Discussion

The NF-κB pathway plays a crucial role in the development of protective immunity to *Toxoplasma* infection (Caamano et al., 1999; Caamano et al., 2000). Studies from our group have shown that the *T. gondii*-established blockade of host apoptosis is dependent on RelA (p65) (Payne et al., 2003) and is linked to the expression of pro-survival and anti-apoptotic genes (Molestina et al., 2003). The present study provides evidence that the NF-κB-dependent transcriptional response of the host cell is governed by: (1) a requirement for an intact host IKK complex at early stages of infection to promote optimal levels of IκBα phosphorylation (Fig. 2) and NF-κB translocation (Figs 3-5); (2) the appearance of a parasite-derived TgIKK activity during active parasite growth (Fig. 1); and (3) an additional level of control by components of the pathway downstream of IKK and TgIKK that regulate the magnitude of expression of selective genes (Fig. 7).

The timing of phospho-IκBα localization at the PVM by 6-9 hours p.i. correlates with a phase of host IKK dampening (Hoffman et al., 2002; Nelson et al., 2004). This suggests a level of temporal regulation that ensures sustained and regulated activation of the NF-κB pathway even in the presence of damped host IKK activity. In the context of infection, such a property might be required for effective manipulation of the cellular responses linked to immune and pro-survival functions by fine tuning the intensity of host gene expression.

The existence of the parasite-derived TgIKK activity suggested that host IKK was expendable in the sustained activation of NF-κB in infected cells. In an earlier study (Molestina et al., 2005), we reported that the ability of *T. gondii* to establish high levels of phospho-IκBα by immunoblot analysis correlated with the reported catalytic activities of IKKα and IKKβ (Cao et al., 2001; Li et al., 1999). This requirement is further reinforced in the present study upon examination of the kinetics of IκBα phosphorylation in response to infection. Accordingly, we find rapid and sustained phosphorylation of IκBα in WT and IKKα$^{-/-}$ cells (Fig. 2A,B). The initial activation is largely absent in IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$ cells, implicating IKKβ as the main contributor to the early response (Fig. 2C,D). More importantly, in the absence of IKKβ, the higher phosphorylation levels of IκBα are not attained later in infection, despite the presence of TgIKK activity at the PVM.

The hierarchy of host IKK and TgIKK activities in phosphorylating IκBα is further reinforced in the temporal analyses of NF-κB translocation and subsequent gene expression. The pattern of p50 and p65 nuclear translocation in the early phase of infection (1-3 hours) of WT cells exhibits the classic damped oscillation profile of the NF-κB signaling module (Hoffman, 2002; Nelson, 2004), which is consistent with the activation of the host IKK signalosome. Translocation of p50 and p65 occurs rapidly in infected WT MEFs and the signal becomes dampened at 6 hours p.i. (Fig. 3). Of note, this 'dampened signal' still represents a net translocation of NF-κB relative to the uninfected control (0 hour time point). The 6 hours time point corresponds to a phase when elevated levels of phospho-IκBα begin to accumulate at the PVM as a consequence of TgIKK activity (Fig. 1). Coincident with this increasing signal, a steady increase in NF-κB translocation is observed as infection progresses to reach a sustained level of activation. It is worth mentioning that this sustained response does not represent complete nuclear translocation of p50 and p65 (Fig. 4). Thus, the fine control of NF-κB translocation in *T. gondii*-infected cells represents a sophisticated level of subversion that is beyond a simple 'on and off' switch.

The pattern of NF-κB translocation in IKK-knockout cells revealed interesting results. Although cells lacking IKKα$^{-/-}$

exhibited no major defect in the phosphorylation of IκBα early in infection, they presented a marked failure to promote both p50 and p65 nuclear translocation at this stage (Fig. 3). This absence of translocation was more expected in cells devoid of IKKβ (i.e. IKKβ$^{-/-}$ and IKKα$^{-/-}$β$^{-/-}$), which had major defects in IκBα phosphorylation at all time points. Thus, we propose that the optimal activity of TgIKK is crucially dependent on the establishment of an 'activation threshold' by the host IKK. In such a scenario, the host IKK serves as the engine driving the initial activation of the pathway, whereas TgIKK activity is required to sustain and modulate the response. Importantly, however, TgIKK alone lacks the capacity to promote robust NF-κB activation, as noted by the absence of nuclear localization of p50 and p65 in IKKα$^{-/-}$β$^{-/-}$ cells (Fig. 4).

The kinetics and magnitude of NF-κB translocation correlated with the activation profile of IL-6 and GRO1. Interestingly, the regulation of these genes displayed fluctuating patterns of expression consistent with waves of NF-κB activation and de-activation. These oscillating patterns of activation fall into two phases characterized by a rapid increase early in infection and a phase of maximal expression coincident with elevated TgIKK activity at the PVM. A functional IKKβ subunit in IKKα$^{-/-}$ cells was not sufficient to induce optimal levels of IL-6 and GRO1 expression (Fig. 7), which might be partially explained by the defect in NF-κB translocation observed early in infection (Fig. 3). In addition, the deficient induction of gene expression in IKKα$^{-/-}$ cells might be attributed to the role of IKKα in phosphorylation of histone H3 and p65, which affects expression of NF-κB target genes (Anest et al., 2003; Yamamoto et al., 2003; Lawrence et al., 2005). Of note, the profound defects in parasite-dependent gene activation owing to IKKα or IKKβ deficiency appear to be selective for early response genes as the expression of the anti-apoptotic gene IAP2 was affected to a much lesser extent (Fig. 7) despite being NF-κB regulated (Wang et al., 1998). These observations suggest that the activation of subsets of anti-apoptotic genes entails the participation of additional signaling networks during infection.

In addition to supporting the essential requirement for IKKα and IKKβ in promoting a robust NF-κB transcriptional response by *T. gondii* infection, our gene array studies revealed the necessity for an intact signalosome in stimulating optimal expression of upstream regulators of the pathway (Fig. 6). Of note, clusters of genes displayed tendencies towards IKKα or IKKβ activation for optimal expression. It is well recognized that distinct stimuli preferentially activate the IKKβ-dependent 'canonical pathway' or IKKα-dependent 'alternative pathway' of NF-κB, resulting in the enhanced expression of specific genes (Bonizzi and Karin, 2004). Nuclear translocation of p50/p65 heterodimers is dependent on the canonical pathway, whereas activation of the alternative pathway results in p52/RelB translocation. A previous study by our group determined the presence of p52/RelB in addition to p50/p65 heterodimers in nuclear extracts of *T. gondii*-infected cells by EMSA (Molestina et al., 2003), supporting the activation of both arms of the NF-κB response by the parasite. As opposed to the early translocation observed with p50/p65, recent studies on the kinetics of p52/RelB translocation show a delayed profile dependent on IKKα as predicted (data not shown). The temporal differences in the activation of the canonical and alternative pathways suggest an additional level of *T. gondii*-derived regulation of NF-κB that might therefore govern parasite virulence.

Population genetic studies indicate that the three major lineages of *T. gondii* (Type I, Type II and Type III) appear to differ in their capacity to promote NF-κB nuclear translocation and gene expression (Robben et al., 2004; Saeij et al., 2005). Our studies have focused on the hypervirulent Type I RH strain of the parasite. We and others find that infection with the RH strain promotes robust phosphorylation of IκBα (Fig. 2) (Molestina et al., 2003; Butcher et al., 2001; Shapira et al., 2005) that is accompanied by the detection of NF-κB in the nucleus by IFA (Fig. 4), immunoblot (Fig. 3) and EMSA (Fig. 5) (Molestina et al., 2003; Kim et al., 2001). More importantly, as confirmed in this study, infection is associated with the increased expression of NF-κB-regulated genes both at early and late time points (Fig. 6) (Blader et al., 2001; Brenier-Pinchart et al., 2000; Denney et al., 1999; Molestina et al., 2003). Finally, the activation of a subset of these genes is not observed in (RelA) p65$^{-/-}$ cells, reinforcing the role of NF-κB in the induction of gene expression (Molestina et al., 2003). Despite this body of evidence, the issue of the activation of NF-κB translocation and gene expression in response to infection with RH strain parasites has recently become an area of controversy (Sinai et al., 2004; Shapira et al., 2004).

In contrast to our observations, the alternative view posits that whereas infection with RH-strain parasites promotes the phosphorylation of IκBα, nuclear translocation of NF-κB subunits is absent (Butcher et al., 2001; Shapira et al., 2002; Shapira et al., 2005). In a recent study, Shapira et al. define the absence of NF-κB translocation, both in early and late infection, relative to the level of NF-κB translocation observed following stimulation with arguably the most potent activator, TNF-α (Shapira et al., 2005). They conclude, on the basis of this comparison, that there is no translocation of NF-κB in response to infection when in fact nuclear labeling of p65 is apparent, albeit at a much lesser extent relative to TNF-α, in infected cells (Shapira et al., 2005).

The proposed blockade of NF-κB subunit translocation, particularly early in infection, is also instituted in macrophages, epithelial cells and fibroblasts, including murine fibroblasts used in our studies (Butcher et al., 2001; Shapira et al., 2002; Shapira et al., 2005). Notably, the lack of a complete nuclear re-distribution of NF-κB subunits mirrors our experience, showing that *T. gondii* infection results in only a fraction of NF-κB accumulating in the nuclei of infected cells (Fig. 4) (Molestina et al., 2003). Regardless, however, the more central issue is whether these levels of nuclear NF-κB are sufficient to drive the parasite-directed activation of gene expression. The data sets from our group (Figs 6, 7) (Molestina et al., 2003) and those of others (Blader et al., 2001; Brenier-Pinchart et al., 2000; Denney et al., 1999; Kim et al., 2001) indicate that Type I RH strain parasites drive and differentially regulate NF-κB-dependent gene expression. By contrast, the claim in support of a failure by *T. gondii* to activate NF-κB-dependent gene expression is not based on the analysis of endogenous genes but the use of a NF-κB–GFP reporter system (Shapira et al., 2005). At the very least, the differences in the results and interpretation point to the need for a thorough and detailed dissection of both the parasite and host determinants in this complex interaction.

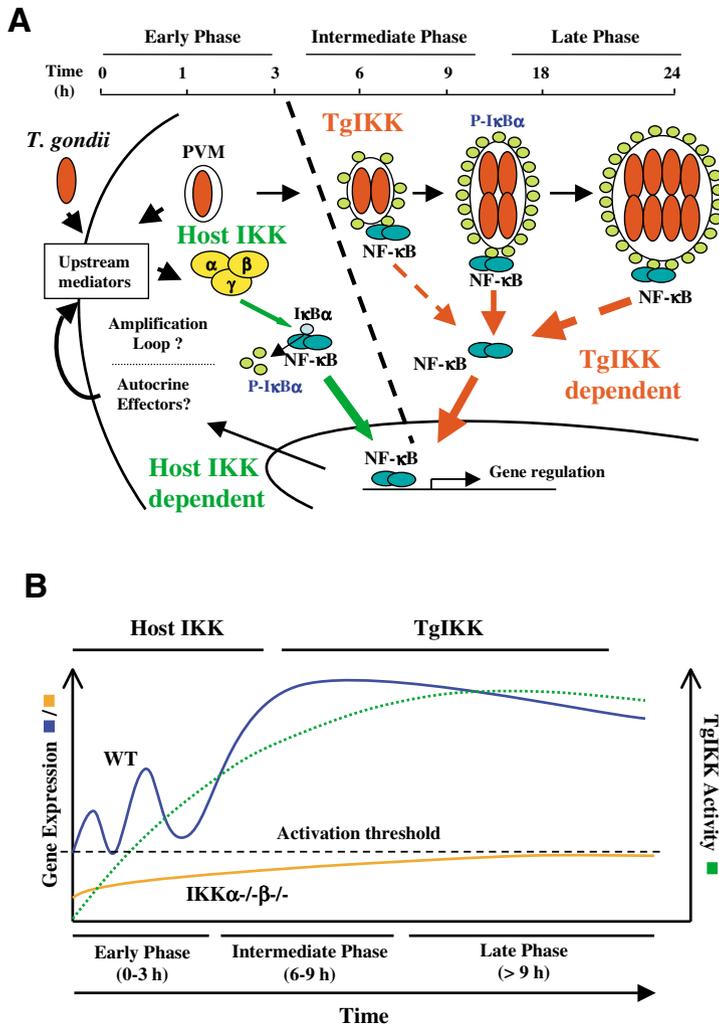The use of MEFs with defined genetic defects in the IKK

A

B

Fig. 8. A model representing the modulation of NF-κB-dependent gene expression by *T. gondii*. (A) Early in infection, the parasite triggers activation of the host IKK complex, resulting in a primary wave of NF-κB translocation and induction of gene expression (early phase). An increase in TgIKK activity at the PVM phosphorylates a subset of IκBα molecules, inducing a secondary wave of NF-κB activation (intermediate phase). The extent of this response is abrogated owing to a disruption in the host IKK signalosome. The magnitude of the NF-κB transcriptional response might also depend on an amplification loop through autocrine effectors (i.e. TNF-α, IL-1), resulting in sustained levels of gene expression at late stages of infection (late phase). (B) Signals deriving from the host IKK signalosome and TgIKK affect the strength and duration of the NF-κB-dependent transcriptional response. Levels of gene expression shown in the blue line are for representation purposes only and do not reflect actual quantitative data. The regulation of specific NF-κB target genes displays oscillatory patterns early in infection (blue line, early phase). An increase in TgIKK activity at the PVM (green dashed line) causes an activation of the response during mid-infection, resulting in sustained levels of NF-κB-dependent gene expression (blue line, intermediate-to-late phases). An impaired IKK complex in the host cell abrogates this response significantly owing to a failure to attain an 'activation threshold' (black dashed line) required by TgIKK for the induction of sustained levels of NF-κB activation (orange line).

the integration of signals deriving from the host IKK signalosome and TgIKK is hypothetically represented in Fig. 8B. The regulation of specific genes displays oscillatory patterns early in infection (Fig. 8B, blue line, early phase), plausibly in a similar fashion as reported with the transient phase of NF-κB activation by pro-inflammatory cytokines (Hoffmann et al., 2002; Schmidt et al., 2003). Following increased TgIKK activity at the PVM (Fig. 8B, green dashed line), an activation of the response during mid-infection results in sustained levels of NF-κB-dependent gene expression (Fig. 8B, blue line, intermediate-to-late phases). An impaired IKK complex in the host cell abrogates this response significantly owing to a failure to attain an 'activation threshold' (dashed line) required by TgIKK for the maintenance of persistent yet regulated levels of NF-κB activation (Fig. 8B, orange line).

Different pathogens have evolved a variety of strategies to evade or exploit the NF-κB pathway to achieve successful co-existence with the host (Tato and Hunter, 2002). Given the bimodal characteristics of NF-κB signaling and selective gene activation by classical activators of the pathway (Hoffmann et al., 2002; Schmidt et al., 2003), our study emphasizes the relevance of analyzing the regulation of host gene expression in a temporal fashion in response to infection. By analogy to *T. gondii*, the temporal profiles of selective NF-κB target genes modulated by infection with other pathogens might display a hierarchical regulation between host- and microbial-derived components.

complex has allowed us to differentiate between host and parasite activities affecting the regulation of the NF-κB response. The molecular events involved in this process are multi-factorial, requiring the orchestrated participation of both host IKK and parasite TgIKK activities to modulate the strength and duration of the NF-κB-dependent transcriptional response. We propose a model where the initial stages of infection trigger activation of the host IKK complex, resulting in a primary wave of NF-κB translocation and induction of gene expression (Fig. 8A). Upstream mediators of this early phase probably involve components of the TLR pathway (Mason et al., 2004b; Mun et al., 2003; Robben et al., 2004; Scanga et al., 2002). As infection progresses, an increase in TgIKK activity at the PVM triggers phosphorylation of a subset of IκBα molecules and a secondary wave of NF-κB activation (Fig. 8A, intermediate phase). The extent of this response is limited in the absence of a functional IKK complex early in infection. The potential role of an amplification loop through an autocrine mechanism (i.e. IL-1, TNF-α) such as that described for the related apicomplexan *Theileria parva* (Dobbelaere and Rottenberg, 2003), cannot be excluded to maintain a persistent state of NF-κB activation at late stages of infection (Fig. 8A, late phase).

The extent of NF-κB-dependent gene expression affected by

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