Supplementary Material.

Material and Methods

Cells. The *T. parva*- and *T. annulata*-transformed cell lines used in this work have been described previously. (S1, S2). Bovine lymph node cells were prepared as described previously (S3).

Digital Confocal Immunofluorescence Microscopy. Antibody reagents: mouse anti-NEMO (Transduction Laboratories), rabbit anti-NEMO (Santa Cruz sc 8330), mouse anti-V5 (Invitrogen), rabbit anti-V5 (MBL), mouse anti-P-IκBα (Santa Cruz, sc 8404), rabbit anti-IKKαβ (Cell Signaling), rabbit anti-IKKα (Santa Cruz, sc 7218), rabbit anti-p65 (Santa Cruz, sc 372), mouse anti-BrdU (G3G4, Developmental studies Hybridoma Bank, University of Iowa), mouse anti-IKKβ (Oncogen OP134), mouse anti-PIM40.3 (ILRI, Nairobi), rabbit anti-Tams (S1) anti-rabbit Texas Red (Vector laboratories), anti-rabbit Alexa Fluor 488 (Molecular Probes), anti-mouse FITC (Jackson Laboratories), anti-mouse Texas Red (Vector laboratories). Immunofluorescence staining: logarithmically growing *T. parva* and *T. annulata*-infected cells were harvested and washed in Leibovitz L15 medium and resuspended at 1 x 10^6 cell ml/ml. Cytospins were prepared using 100 μl of cell suspension. Cells were fixed in 100% methanol at -20°C for 5 min and permeabilized in 0.2% TritonX100 for 10 min at RT. First antibodies were applied in the appropriate concentrations for 1 h at RT. Cells were washed subsequently twice with PBS and incubated with a combination of Texas Red-labeled anti-rabbit antibodies and a FITC labeled anti-mouse antibody. Alternatively, a combination of Texas Red-labeled anti-mouse antibodies and Alexa 488-labelled anti-rabbit antibodies were used to stain the cells. Cells were washed twice in PBS and nuclei were stained with Hoechst 33258 for 1 min before slides were mounted. Slides were analyzed using a Nikon Eclipse 800 microscope. Images were produced by digital confocal imaging using Openlab software (relaease 2.0.8, Improvision).

Transfection of plasmids. Constructsencoding wild type and dominant-negative mutants were transfected as described by Heussler et al. (S2) except that plasmids were mixed with 600 μl of cell suspension (2 x 10^7 cells/ml) and pulsed at 1640 μF, 300V, 1500 Ohm. The following plasmids were used: pcDNA3-TRAF2 and pcDNA3-dn-TRAF2 (S4) pME-Flag-TRAF6, pME-Flag-TRAF6(C85A H87A), pEF-HA-ubc13, pEF-HA-ubc13(C87A) (S5).
pRK5myc-POSH, pRK5myc-deltaPOSH (S6), pSFFV-YopJ-Flag, pSFFV-YopJ-Flag (C172A) (S7), pCDNA3.Flag-Nak, pCDNA3.Flag Nak/KM (S8), SRα3-WTMEKK-1, SRα3-WTMEKK-1(K432M) (S9), pCMV-HA-TAK1, pCMV-HA-TAK1(K63W) (S10). Expression vectors for wild type A20, wild type and dominant-negative mutant NIK, wild type and dominant-negative mutant RIP, wild type and dominant-negative mutant IKK1, wild type and dominant-negative mutant IKK2, wild type NEMO and delta-NEMO have been described elsewhere (S11). The cDNA of RIP2 was cloned by PCR from a human macrophage cDNA library, linked to an N-terminal HA tag and inserted to the pCDNA vector. A kinase-dead mutant of the protein (K47A) was derived by PCR. The plasmid pCDNA3.IS32A was described previously (S12) and used as a positive control for NF-κB inhibition.

**Luciferase assay.** For this assay, 10 μg of NF-κB3-luciferase plasmid and 0.5 μg of the tk-Renilla luciferase plasmid (Promega) were co-transfected with 10 μg of the expression vectors encoding wild type and dominant-negative mutants or empty control vector. After transfection, cells were cultured for 16h and luciferase assays were performed using the Promega Dual Luciferase Reporter Assay System. Transfection efficiency was standardized on the basis of constitutive renilla luciferase activity.

**NBD-inhibitor peptide experiments.** *T. parva*-transformed T-cells were incubated for 3 h in the presence of 200 μM cell-permeable NEMO-Binding Domain Binding Peptide (Calbiochem) or its negative control peptide. Cytospin preparations were made and stained with anti-NEMO antibodies to visualize parasite-associate IKK signalosomes and with anti-P-IκBα to monitor the state of IKK signalosome activation.

**Immunoblotting and BrdU incorporation** were performed as described in Heussler et al, (S2).
**Supplemental Figure 1.** Parasite-associated IKK foci can also be detected by antibodies directed against IKK2 and NEMO (red). IKK foci cannot be detected in cells from which the parasite has been eliminated by treatment for 2 days with the theilericidal drug BW720c (marked with *). Parasite and host cell nuclei were visualized using the DNA stain Hoechst 33258 (blue).
Supplemental Figure 2. Upon treatment with the theilericidal compound BW720c, the parasite is eliminated and NF-κB DNA binding activity downregulated. (A) *Theileria* schizont proteins gradually disappear from the host cell upon addition of BW720c to the culture medium. Total cell lysates were prepared from *T. parva*-transformed cells cultured in the absence (Tp) or presence of BW702c for 1 to 3 days. Immunoblot analysis was carried out using anti-tubulin antibodies to detect host cell tubulin; parasite heat shock protein 70 was detected using a parasite-specific anti-hsp70 antibody. (B) lysates from the same cells were analyzed with antibodies directed against IKK1, IKK2 and NEMO. Lysates from uninfected lymph node cells (L) were loaded as a control.

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Supplemental Figure 3. NF-κB nuclear translocation is downregulated upon elimination of the parasite. (A) Electrophoretic mobility shift assay using [32P]-labeled κB-specific oligonucleotides carried out using nuclear extracts prepared from T. parva-transformed cells and cells cured of the parasite by BW720c treatment for 1 to 3 days; ‘C’ represents a competition experiment with a 100x excess of unlabeled oligonucleotide. (B) In cells cured of the parasite, NF-κB is largely absent from the nucleus. Cytospin preparations of cells cultured in the presence of the theilericidal compound BW720c for 3 days were stained with an antibody directed against the p65 subunit of NF-κB (green) and examined by digital confocal microscopy. Parasite and host cell nuclei were visualized using the DNA stain Hoechst 33258 (blue).
**Supplemental Figure 4.** The presence of parasite-associated IKK signalosomes is strongly reduced in mitotic cells. (A) Confocal immunofluorescence picture of two *T. parva*-transformed cells; Hoechst DNA staining (blue) shows the condensed chromosomes of the left cell in mitosis and a normal interphase nucleus of the cell on the right. IKK signalosomes visualized using anti-IKK antibodies (red) are only detectable in the interphase cell. (B) Cells were arrested in mitosis by treatment with colchicine (10 µM) and the percentage of cells in interphase or mitosis in which IKK signalosomes could be detected at a standard magnification of x400 was determined. Standard deviations represent the error for three separate preparations. (C) Confocal immunofluorescence picture of two *T. parva*-transformed cells stained with Hoechst DNA stain (left panel); one of the two cells is undergoing DNA synthesis (BrdU⁺, green), but IKK signalosomes (red) can be detected in both cells. (D) The percentage of cells containing parasite-associated IKK signalosomes was determined for cells undergoing DNA synthesis and cells in G1 or G2. Cells in mitosis were not counted.
Supplemental Figure 5. Inhibition of classical NF-κB pathways by overexpression of different dominant-negative mutants and wt-A20. κB-dependent luciferase reporter gene assays were performed on freshly isolated bovine lymph node cells transfected with different dn-mutant constructs or the wt-A20 construct as indicated. Sixteen hours after transfection, cells were stimulated for 8h with either bovine TNFα (50 ng/ml), bovine IL-1 (50ng/ml) or LPS (100ng/ml) and relative luciferase activity was measured. To test dn-POSH, cells were starved after transfection by FCS withdrawal and then stimulated for 8 h by addition of 20% FCS. To demonstrate the inhibitory capacity of dn-IKK1, cells were stimulated by NIK overexpression (S13) in the presence or absence of dn-IKK1.
Supplemental references


