The Viral Protein A238L Inhibits Tumor Necrosis Factor Alpha (TNF-α) Expression through a CBP/p300 Transcriptional Coactivators Pathway.

Running Title: A238L inhibits TNF-α through a CBP/P300 pathway

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Abstract

African swine fever virus (ASFV) is able to inhibit TNF-α-induced gene expression through the synthesis of A238L protein. This was shown by the use of deletion mutants lacking the A238L gene from the Vero cell-adapted Ba71V ASFV strain and from the virulent isolate E70. To further analyze the molecular mechanism by which the viral gene controls TNF-α, we have used Jurkat cells stably transfected with the viral gene to identify the TNF-α regulatory elements involved in the induction of the gene after stimulation with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (Ion). We have thus identified the cyclic AMP-responsive element (CRE) and κ3 sites on the TNF-α promoter as the responsible of the gene activation, and demonstrate that A238L inhibits TNF-α expression through these DNA binding sites. This inhibition was partially reverted by overexpression of the transcriptional factors NFAT, NFκB, and c-Jun. Furthermore, we present evidence that A238L inhibits the activation of TNF-α by modulating NFκB, NFAT and c-Jun transactivation through a mechanism that involves CBP/p300 function, since overexpression of these transcriptional coactivators recovers TNF-α promoter activity. Taken together, these results establish a novel mechanism in the control of TNF-α gene expression by a viral protein that could represent an efficient strategy used by ASFV to evade the innate immune response.

Abbreviations: Footnote (3)

Introduction

The tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine able to inhibit viral infection. TNF-α is secreted by multiple cell types such as macrophages and lymphocytes after viral infection or stimulation through cell surface receptors, and induces the synthesis of a wide array of molecules which mediate the inflammatory response and regulate immune cell function (1). TNF-α has been demonstrated to act as a potent antiviral cytokine that is directly cytotoxic to cells infected with both RNA and DNA viruses (2), and has been shown to inhibit the replication of herpes simplex virus, varicella-zoster virus, cytomegaloviruses and African swine fever virus *in vitro* (3-5).

The promoter sequences required for induction of the human TNF-α gene by a variety of stimuli, including viruses, phorbol esters, calcium ionophore or LPS, have been identified, and serve as the primary control point of the regulation of TNF-α production (6-9). These studies have established that NFκB, NFAT, ATF-2, Jun, Ets/Elk, and Sp-1 proteins and the CREB binding protein (CBP) and p300 coactivator proteins are involved in the specific regulation of the human TNF-α gene, depending on the cell type and the stimuli (6, 10-13).

In activated T cells, TNF-α is an immediately early gene induced by calcium fluxes through a calcineurin-dependent process. This activation requires a cyclic AMP response element (CRE) (14), which binds ATF-2/Jun, and two NFAT-binding sites, the -76-NFAT and κ3-NFAT sites (6). In macrophages, the basal and PMA-induced TNF-α promoter activity is very similar to that observed in T lymphocytes. Expression of c-Jun in U937 human macrophage and in MLA 144 T cell lines caused consistent augmentation of promoter activity, indicating that AP-1 plays an important role in transcriptional regulation of the TNF-α promoter (8).

African swine fever virus (ASFV), the sole member of the *Asfarviridae* family (15), encodes a protein, A238L, which has been described to inhibit calcineurin phosphatase activity (16). A238L also down-regulates the activation of the NFκB and NFAT transcription factors, both when expressed in Jurkat cells or during ASFV infection (17, 18). In a previous report, we have shown that ASFV is thus able to control the transcriptional activation of immunomodulatory genes dependent on NFκB and NFAT pathways, such as cyclooxygenase-2 (COX-2) (18).

Here we have investigated the ability of ASFV to inhibit TNF-α gene expression through the synthesis of A238L protein. To achieve this, we have used deletion mutants lacking the A238L gene from the Vero cell-adapted Ba71V ASFV strain and from the virulent isolate E70. Using these tools, we have characterized events involved in TNF-α gene induction following infection of Vero cells or porcine macrophages, the natural target of the infection. To further analyze the function of A238L on the control of TNF-α, we have used Jurkat cells stably transfected with the viral gene to identify the TNF-α promoter regulatory elements involved in the induction of the gene after stimulation with phorbol 12-myristate 13-acetate plus calcium ionophore (PMA/Ion) and the modulation by A238L. We have identified the CRE and κ3 sites on the TNF-α promoter as the responsible of the gene activation in our system, and demonstrate that A238L inhibits the TNF-α expression through these sites. This inhibition was reverted by overexpression of NFAT, NFκB, and c-Jun, but not by c-Fos. Furthermore, we present preliminary evidence that the mechanism by which A238L inhibits the activation of TNF-α involves the modulation of transactivation of NFκB, NFAT and c-Jun, through the transcriptional coactivators CBP and p300. Taken together, these results establish a novel mechanism in the control of TNF-α gene expression by a viral protein that could represent an efficient strategy used by ASFV to evade the innate immune response.

Materials and Methods

Cell culture, viruses and reagents

Vero (African green monkey kidney) cells and COS-7 (African green monkey kidney) cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (Gibco). Jurkat human leukemia T cell line was obtained from the ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Porcine alveolar macrophages were obtained by broncho-alveolar lavage of pigs and cultured in DMEM supplemented with 10% homologous swine serum (19). All media were supplemented with 2 mM L-glutamine, 100 U of gentamicin per ml and non essential amino acids. Cells were grown at 37ºC in 7% CO_2 in air saturated with water vapor. Jurkat and Vero cells were stimulated by phorbol 12-myristate 13-acetate (PMA; Sigma) at 15 ng/ml and A23187 calcium ionophore (Ion; Sigma) at 1 μM. Cyclosporin A (CsA, Sandoz, 100 ng/ml) was added 1 h before the addition of PMA and Ion. The Vero-adapted ASFV strain Ba71V was propagated and titrated by plaque assay on Vero cells as described (20). The virulent ASFV strain E70 was propagated and titrated by plaque assay on swine alveolar macrophages as described (19, 21).

ASFV A238L deletion mutants construction

The A238L-defective mutant ΔA238L viruses were obtained by insertion of the *Escherichia coli* β-glucuronidase (β-gus) gene into the viral A238L open reading frame. The recombinant Ba71VΔA238L virus was obtained as previously described (18). The recombinant E70ΔA238L was obtained using the same transfection-infection protocol but infecting COS-7 cell monolayers with the viral strain E70.

The lack of gene in the recombinant E70ΔA238L virus was assessed by Southern blot hybridization. Briefly, DNA samples obtained from purified E70 and ΔA238L viruses were digested with the restriction endonuclease *Eco* RI, subjected to electrophoresis in agarose gels, and transferred to nylon membranes following standard procedures (22). The DNA probes, specific for the β-gus and A238L genes, and for the *Sal* I I*'* fragment of E70 genome were labeled with a DIG DNA labeling kit (Boehringer Mannhein) using manufacturer's instructions, and hybridizations were done as described elsewhere (23).

mRNA analysis

Total RNA was prepared from Jurkat-pcDNA, Jurkat-A238L, ASFV-infected porcine macrophages or ASFV-infected Vero cells by the TRIzol reagent RNA protocol (Invitrogen). Total RNA (1 μg) was reverse transcribed into cDNA by the RevertAid First Strand cDNA synthesis kit (MBI Fermentas), and used for PCR amplification with the addition of Taq DNA polymerase (Roche) following the manufacturer's instructions. Specific primers used in PCR reactions were porcine TNF-α (forward: 5'-CTCTTCTGCCTACTGCACTTCGAGG-3' and reverse: 5'-CTGGGAGTAGATGAG-GTACAGCCCA-3'), porcine GAPDH (forward: 5'-AGCTTGTCATCAATGGAAAG-G-3' and reverse: 5'-AGAAGCAGGGATGATGTTCTG-3'), human TNF-α (forward: 5'-TCAGATCATCTTCTCGCACCC-3' and reverse: 5'-GACTCGGCAAAGTCGAG-ATAG-3'), human β-actin (forward: 5'-GAGAAGATG-ACCCAGATCATG-3' and reverse: 5'-TCAGGAGGAGCAATGATCTTG-3'), viral A238L (forward: 5'-CGCGC-GTCTAGATTACTTTCCATACTTGTT-3' and reverse: 5'-GCGCGCAAGCTTATG-GAACACATGTTTCCA-3'). The PCR reactions were performed by 30 cycles of

denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min. Amplified cDNAs were separated by agarose gel electrophoresis.

Western blot analysis

Cytosolic and nuclear extracts from Jurkat-pcDNA and Jurkat-A238L cells, unstimulated or stimulated with 15 ng/ml of PMA plus 1 μM Ion and treated or not with 100 ng/ml of CsA were prepared as described previously (18). To prepare whole-cell extracts, mock-infected or ASFV-infected Vero cells, and Jurkat-pcDNA and Jurkat-A238L cells were washed twice with PBS and lysed in radio immunolabeling protein assay (RIPA) buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 and 0.25% Na-deoxycholate, and supplemented with protease inhibitor cocktail tablets (Roche). In each case, protein concentration was determined by the bicinchoninic acid (BCA) spectrophotometric method (Pierce). Cell lysates (30 μg of protein) were fractionated by SDS-12% polyacrylamide gel electrophoresis, electrophoretically transferred to an Immobilon extra membrane (Amersham), and the separated proteins reacted with specific primary antibodies raised against A238L (generated as described in (17)), NFκB-p65 (sc-109, Santa Cruz Biotechnology), c-Jun (sc-45, Santa Cruz Biotechnology), JNK1 (sc-474, Santa Cruz Biotechnology), and β-actin (H-196, Santa Cruz Biotechnology). Membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), followed by chemiluminescence (ECL, Amersham Biosciences) detection by autoradiography.

Quantitation of TNF-α in culture supernatants

Alveolar porcine macrophages were infected with the E70 wt and E70ΔA238L viruses at a multiplicity of infection (MOI) of 5 plaque forming units per cell (pfu/cell), and supernatants were recovered at the indicated post-infection times. TNF-α protein levels were measured using "Quantikine" (R&D Systems) enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions.

Plasmid constructs

Human TNF-α promoter constructs containing the full-length promoter sequence fused to firefly luciferase reporter gene, named pTNF(-1311)luc, or the different 5' deletion mutants, named pTNF(-751)luc, pTNF(-528)luc, pTNF(-120)luc, pTNF(-95)luc and pTNF(-36)luc were generated as described (8). The full-length human NFATc (p1SH107c) expression plasmid (24) was a generous gift from Dr. G. Crabtree. The p65 expression plasmid pcDNA3-p65 was a gift from Dr. J. Alcamí. The pRSV-c-Jun and the pRSV-c-Fos expression plasmids have been previously described (25). The pcDNA-A238L expression plasmid was generated by cloning the A238L open reading frame from Ba71V viral strain of ASFV into the pcDNA3.1 mammalian expression vector (Invitrogen). The GAL4-luciferase construct (pGAL4-Luc) contains five GAL4 DNA consensus binding sites derived from the yeast GAL4 gene fused to luciferase reporter gene (26). The pGAL4-hNFAT1 construct containing the first 1-451 amino acids of human NFAT1 fused to the DNA-binding domain of yeast GAL4 transcription factor was originated as described previously (27). The pGAL4-p65 construct has the yeast GAL4 DNA binding domain fused to the carboxyl-terminal transactivation domain of p65, and was generated as described (28). The pGAL4-c-Jun was generated as described (29). The CREB Binding Protein (CBP) expression plasmid pRC/RSV-CBP-HA was a

generous gift from Dr. A. Harell-Bellan and generated as described (30), and the p300 expression plasmid pCMVβ-p300-HA was a generous gift from Dr. Joan Boyes and generated as described (31).

Transfection and luciferase assays

Generation of A238L stably expressing Jurkat cells was done as described in (18). A238L stably expressing Vero cells were generated using the same protocol. These cellular lines were named Vero-pcDNA and Vero-A238L.

Jurkat-pcDNA and Jurkat-A238L cells, Vero cells, and porcine macrophages were transfected with 250 ng of specific reporter plasmids per 10^6 cells using the LipofectAMINE Plus Reagent (Invitrogen) according to the manufacturer's instructions and mixing in Opti-MEM (Invitrogen). In cotransfection assays, 0.05-0.5 μg of the corresponding expression plasmid per 10^6 cells were added. Sixteen hours after transfection, Jurkat-pcDNA and Jurkat-A238L cells were stimulated with 15 ng/ml of PMA plus 1 μM Ion during 4 h, Vero cells were infected with Ba71V or Ba71VΔA238L at a MOI of 5 pfu/cell, and porcine macrophages were infected with E70 or E70ΔA238L at a MOI of 5 pfu/cell. At the indicated times, cells were lysed with 200 μl of Cell Culture Lysis Reagent (Promega) and microcentrifuged at full speed for 5 min at 4ºC, and 20 μl of each supernatant was used to determine firefly luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Results were expressed as the luminescence units after normalization of protein concentration determined by the BCA method. Transfection experiments were performed in triplicate, and the data presented as the mean of the relative luciferase units (RLU) (mean ± S.D.).

Immunofluorescence and confocal microscopy

Vero cells were grown on cover slips to $2x10^5$ cells/cm^2 and then stimulated with 15 ng/ml of PMA plus 1 μM Ion during the different times indicated in Fig. 6. The cultures were rinsed 3 times with PBS and fixed with cold 99.8% methanol (Merck) for 15 min at -20ºC, before rehydrating twice with PBS and blocking with 1% BSA in PBS for 10 min at room temperature. The cells were incubated during two hours with the specific antibody against NFATc2 (sc-7295, Santa Cruz Biotechnology), rinsed extensively with PBS and then incubated with the secondary antibody (Alexa, Molecular Probes) for 1 h at room temperature in the dark. Finally, the cells were rinsed successively with PBS, distilled water and ethanol, and mounted with a drop of Mowiol on a micro slide. Visualization of stained cultures was performed under a fluorescence Axioskop2 plus (Zeiss) microscope coupled to a color CCD camera or to a Confocal Microradiance (BioRad) equipment. Images were digitalized, processed and organized with Metamorph, Lasershap2000 v.4, Adobe Photoshop 7.0, Adobe Illustrator 10, and Microsoft PowerPoint SP-2 software.

Solid-phase in vitro phosphorylation kinase assay

We used 2 μg of GST-c-Jun (sc-4113, Santa Cruz Biotechnology) as the substrate for in vitro phosphorylation in which immunoprecipitated JNK1 from Jurkat-pcDNA or Jurkat-A238L were assayed. Whole-cell extracts from 10^7 Jurkat-pcDNA or Jurkat-A238L cultured in the absence or presence of 15 ng/ml of PMA plus 1 μM Ion during 30 minutes were prepared. The cells were lysed in RIPA buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 1% NP-40, and supplemented with phosphatase inhibitors (1 mM $NaVO_3$, 10 mM NaF, and 10 mM Na_2MoO_4) and protease inhibitors

(0.5 mM phenylmethylsulfonil fluoride, 1 μg of pepstatin, 2 μg of leupeptin, and 2 μg of aprotinin per ml).

Cleared extracts were incubated for 18 h with 1 μg of specific antibody against JNK1 (sc-474, Santa Cruz Biotechnology) to immunoprecipitate JNK1. Immunoprecipitates were finally resuspended in kinase buffer containing 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 μM ATP, and 1 μCi of [γ^{32}P]ATP (specific activity, 3,000 Ci/mol) supplemented with phosphatase inhibitors and mixed with the recombinant c-Jun. After 30 min at 30ºC, the kinase reaction was terminated by washing with TNT buffer containing 20 mM Trizma base (pH 7.5), 200 mM NaCl and 1% Triton X-100, and supplemented with protease inhibitors cocktail tablets (Roche). Phosphorylated proteins were separated in a SDS-12% polyacrylamide gel electrophoresis, dried and developed by autoradiography.

Results

A238L inhibits TNF-α promoter activity and transcription during ASFV infection in Vero cells and porcine macrophages

TNF-α transcription is regulated by several transcription factors such as NFκB, NFAT, ATF-2, Jun, Ets/Elk, and Sp-1 proteins. Since the viral protein A238L has been described as an inhibitor of NFκB (17), NFAT (16, 18) and calcineurin phosphatase (16), we have explored the possibility that this protein could inhibit TNF-α expression. To assess this, we have used the ASFV A238L deletion mutant, designated Ba71VΔA238L, obtained from the Ba71V viral strain as previously described (18). The absence of A238L protein in Ba71VΔA238L-infected Vero cells was corroborated by Western blot analysis with cellular extracts from Ba71V and Ba71VΔA238L-infected cells using a specific antibody against A238L protein. Fig 1A shows the lack of expression of the viral protein in Ba71VΔA238L-infected Vero cells. In contrast, a band corresponding to the protein A238L could be detected from 6 hours post infection (hpi) in the cells infected with the parental virus.

To investigate the role of A238L in the control of TNF-α transcription during ASFV infection, Vero cells were first transfected with the plasmid pTNF(-1311)luc, which contains the luciferase reporter gene under the control of the full length sequence of the human TNF-α promoter. Sixteen hours after transfection, cells were infected either with the parental Ba71V or with ΔA238L viruses (MOI of 5 pfu/cell), and, at the indicated times after infection, luciferase activity was measured in cell extracts. As shown in the Fig. 1B, a slight induction of TNF-α promoter activity was detected after 12 hpi with the wild type virus. However, a much higher activity of the TNF-α promoter was observed from 6 hpi in cells infected with the deletion mutant virus. This

result indicates that A238L efficiently down regulates TNF-α promoter activation during ASFV infection in Vero cells.

We then compared the induction of TNF-α mRNA expression after infection of Vero cells with the parental Ba71V ASFV strain or with the recombinant Ba71VΔA238L to determine the role of A238L in TNF-α gene expression during ASFV infection. As shown in Fig 1C, TNF-α mRNA was identified in Vero cells after infection with the Ba71VΔA238L, while no detectable TNF-α specific mRNA was found after the infection with Ba71V wild type. As expected, no specific A238L mRNA was detected in Ba71VΔA238L-infected cells.

ASFV replicates mainly in macrophages and monocytes in vivo. This fact has been considered to play a critical role in the pathogenesis of the disease, since macrophage-derived cytokines strongly determine the development of inflammatory responses against infection. Since tissue macrophages appear to be the main source of TNF-α, and previous studies have demonstrated impairment of chemotactic activities and toxic oxygen radicals release in macrophages infected with different strains of ASFV (32), the role of A238L in the control of TNF-α in this cellular type is an important issue to address. To study this point, we have generated an ASFV A238L deletion mutant (E70ΔA238L) from the virulent strain E70, which has been shown to induce a strong infection in these cells (21). Recombinant virus expressing the β-gus gene was purified and genomic DNA from wild-type and ΔA238L virus was analyzed by Southern blot, using digoxigenin-labeled DNA probes. As shown in Fig. 2A, DNA fragments of predicted size were observed in both viruses when probed with the parental DNA fragment *Sal* I I′, while the β-gus gene probe hybridized only with DNA

from E70ΔA238L. As expected, the A238L gene probe failed to hybridize with DNA from E70ΔA238L.

We next studied by RT-PCR the expression of A238L gene in porcine macrophages infected with the recombinant virus E70ΔA238L or with the wild type virus. As shown in Fig 2 B, no A238L mRNA was detected in extracts from macrophages infected with E70ΔA238L. When we used these samples to amplify the TNF-α specific mRNA, relatively higher levels of TNF-α mRNA were detected compared to those obtained after Ba71V infection of Vero cells (Fig. 1 B), either due to the different cell type or to the fact that virulent isolates of ASFV are better inducers of TNF-α (33). More importantly, the levels of TNF-α mRNA after infection of macrophages with the recombinant virus E70ΔA238L were much higher than those induced by the parental virus, demonstrating an inhibitory role of A238L in the TNF-α expression during ASFV infection in macrophages (Fig. 2B).

The activity of the TNF-α promoter was also studied as above described in swine macrophages during the infection with E70 or E70ΔA238L, revealing a similar pattern to that obtained (Fig. 1B) during the infection wit Ba71V or Ba71VΔA238L in Vero cells. As shown in Fig. 2C, a higher activity could be detected after 12 hpi in cells infected with the deletion mutant, in correspondence to the levels of TNF-α mRNA obtained.

To evaluate whether the increase of TNF-α mRNA observed after E70ΔA238L infection corresponds to an increase in the TNF-α protein secretion, we quantified the amount of porcine TNF-α in supernatants collected from the infected macrophages. Fig. 2D shows the almost undetectable TNF-α production after the infection with the E70 wild type virus. In contrast, the infection with the deletion mutant virus induces a

substantial increase in TNF-α production from 12 hpi. Taken together these results show the ability of A238L to control the activation of the TNF-α promoter and protein secretion, representing an important mechanism to evade the immune response by ASFV.

A238L regulates TNF-α transcription and promoter activity in Jurkat cells through k3 and CRE sites

To further analyze the mechanism by which A238L regulates TNF-α expression out of the context of ASFV infection, we have generated Jurkat cells that stably express the A238L gene by transfection with pcDNA-A238L expression plasmid, followed by selection using G418 as previously described (18). Stimulation with PMA/Ion increased the levels of TNF-α mRNA in Jurkat-pcDNA cells, which were significantly higher than those found in Jurkat-A238L (Fig. 3A). Thus, expression of A238L strongly decreased TNF-α transcription upon PMA/Ion treatment in Jurkat cells, in concordance with the up regulation of TNF-α mRNA levels by the absence of A238L during viral infection.

To analyze whether the inhibition of TNF-α mRNA levels by A238L correlated with a decrease in the transcriptional activity mediated by the TNF-α promoter, Jurkat-pcDNA or Jurkat-A238L cells were transfected with the plasmid pTNF(-1311)luc, which contains the luciferase reporter gene under the control of the full length sequence of the human TNF-α promoter. As shown in Fig. 3B, and in parallel with the down regulation of TNF-α mRNA levels, ectopic A238L expression strongly decreased the transcription driven by this construction.

In order to investigate the molecular mechanism by which A238L regulates TNF-α promoter activity, we have explored the TNF-α promoter sequences required for

the transcriptional inhibition of TNF-α in Jurkat-A238L. Jurkat-pcDNA and Jurkat-A238L cells were transfected with different 5′ deletions of the TNF-α promoter, pTNF(-751)luc, pTNF(-528)luc, pTNF(-120)luc, pTNF(-95)luc and pTNF(-36)luc. Sixteen hours after transfection cells were cultured in the absence or presence of PMA/Ion for 4 h and assayed for luciferase activity (Fig. 4).

The human TNF-α gene promoter contains three NF-κB consensus sequences, named κ1, κ2, and κ3. Consistent with studies in other cell types and with other inducers, deletion of κ1 and κ2 does not affect the inducibility of the gene, and 120 nucleotides upstream of the TNF-α transcription start site are sufficient for maximal inducibility of the gene. The κ3 site, but not the κ1 or κ2 sites, is included in the pTNF(-120)luc reporter construct. The κ3 site, in contrast to the κ1 and κ2 sites, has been implicated in the regulation of the gene in a variety of cell types (10). It has been described that the κ3 site binds NFAT and NFκB, and that an immediately adjacent CRE site binds ATF-2 and c-Jun proteins. The results presented in Fig. 4 show that A238L expression decreased about 50-70% the transcription driven by -751, -528 and -120 TNF-α promoter constructs. Deletion up to -95 nucleotides significantly reduced activation of TNF-α promoter activity by PMA/Ion, although deletion of the -95 to -36 region of the TNF-α completely abrogated TNF-α inducibility by PMA/Ion. These results indicate not only that κ3 and CRE sites seem to be essential for full transcriptional activation of the TNF-α human gene in our system, but also that the ectopic expression of A238L results in more than 50% reduction of the activity of the TNF-α promoter mediated by this region.

NFAT, p65 and c-Jun, but not c-Fos, participate in the transcriptional activation of the TNF-α gene and are regulated by A238L

The above results show that TNF-α promoter induction was strongly inhibited by the expression of the A238L viral gene in Jurkat cells through κ3 and CRE sites. To characterize the transcription factors that are able to bind to this region and that could be involved in the down-regulation induced by the viral protein, we have cotransfected expression plasmids for NFAT/c2, NFκB (p65), c-Jun and c-Fos, together with the pTNF(-120)luc, into Jurkat-pcDNA or Jurkat-A238L cells. As shown in Fig. 5A, overexpression of NFAT/c2 increased the activity of the promoter after stimulation with PMA/Ion in a dose-dependent manner, corroborating the involvement of NFAT in the modulation of TNF-α expression by the viral protein. Similarly, increased doses of p65-NFκB strongly cooperated with PMA/Ion to activate the TNF-α promoter in Jurkat-A238L cells (Fig. 5B), demonstrating the involvement of the NFκB pathway in the mechanism of TNF-α inhibition by the viral protein. Next, we cotransfected the pTNF(-120)luc construct along with increasing amounts of c-Jun expression plasmid, a member of the AP-1 protein family. As shown in Fig. 5C, cotransfection of pRSV-c-Jun also counteracted the inhibition of the TNF-α promoter activity mediated by A238L. However, increasing doses of c-Fos, an AP-1 family member which is not required for the inducibility of the TNF-α promoter in PMA/Ion-activated T cells, were unable to recover the inhibition mediated by A238L, corroborating the specific involvement of NFAT, NFκB (p65) and c-Jun in this process.

The Inhibition of TNF-α by A238L does not involve NFAT or NFκB nuclear translocation or c-Jun phosphorylation

As we described above, the overexpression of NFAT, NFκB and c-Jun restored partially the TNF-α expression inhibited by A238L, suggesting that the mechanism by which A238L mediates this inhibition in Jurkat cells involves, at least, the activity of these transcription factors.

We have previously described that the mechanism responsible for the A238L-mediated inhibition of NFAT does not imply dephosphorylation or translocation of this transcription factor to the nucleus upon treatment of Jurkat-pcDNA or Jurkat-A238L with PMA/Ion or during ASFV infection (18). To confirm these results in a different cellular system, we have analyzed the nuclear shuttling of NFATc2 both in Vero-pcDNA and Vero-A238L cells by confocal microscopy using a specific anti-NFAT antibody. As shown in Fig. 6A, nuclear translocation of NFAT increased after 30 min of stimulation with PMA/Ion both in the absence and in the presence of A238L expression. This translocation was not observed in cells pretreated with CsA, which prevents NFAT nuclear activation through the inhibition of calcineurin phosphatase. Taken together, these findings suggest that the inhibition of NFAT by A238L is not likely the result of preventing the NFAT translocation to the nucleus in Vero cells.

On the other hand, it is widely accepted that the nuclear translocation is a hallmark of the transcriptional activation of NF-κB and that the intracellular localization of this transcription factor is governed by IκBs. We have previously described that A238L inhibits NFκB activation (17). Here, we have analyzed the presence of p65 in subcellular fractions from Jurkat-pcDNA and Jurkat-A238L after PMA/Ion stimulation to determine whether the control of NFκB activation is due to the inhibition of p65 translocation to the nucleus or to an alternative mechanism. As shown in Fig. 6B, the level of p65 detected in the cytoplasm from both Jurkat-pcDNA and Jurkat-A238L was

similar from 0 to 90 min after PMA/Ion stimulation. Interestingly, the expression of A238L did not impair the translocation of p65 to the nucleus of the stimulated cells.

As mentioned above, the region involved in the regulation of TNF-α transcription contains a CRE binding site, which binds ATF-2/Jun heterodimer and forms a composite element with the κ3 site (6, 34, 35). ATF-2/Jun proteins become transcriptionally active upon phosphorylation by the p38 and Jun kinase 1 (JNK1), members of the mitogen-activated protein kinase family (36). JNK1, whose activity can be augmented by increasing levels of intracellular calcium, binds to the N-terminal region of c-Jun and phosphorylates it at Ser 63/73 (37). We next evaluated the effect of A238L on c-Jun expression and JNK1 activity by analysis of extracts from Jurkat-pcDNA or Jurkat-A238L. Fig. 6C shows the c-Jun expression detected by Western blot with specific antiserum against human c-Jun. Since c-Jun activity depends mainly on its phosphorylation, the effect of A238L on the level of phosphorylated c-Jun was examined through a solid-phase in vitro phosphorylation kinase assay (Fig. 6D), in which immunoprecipitated JNK1 from both Jurkat-pcDNA and Jurkat-A238L, non-stimulated or stimulated with PMA/Ion, was incubated with purified GST-c-Jun as substrate. These results show that the presence of A238L does not inhibit c-Jun expression or JNK1 activity, suggesting that these are not the mechanisms used by the viral protein to down regulate the TNF-α transcription.

A238L down-regulates the transactivation function of NFAT, p65 and c-Jun

The above results indicate that A238L is acting on the NFAT and NFκB activity without altering their nuclear translocation. Besides, A238L does not affect either c-Jun expression or JNK-dependent activation. Recent evidence indicates that activation of NFAT does not only involve its nuclear translocation, but also the intrinsic function of

the transactivation domain which is located at the N terminus (38). To study the regulation of the transactivating function of NFAT by A238L, Jurkat-pcDNA or Jurkat-A238L were transfected with a GAL4-luc reporter plasmid along with a construct (GAL4-NFATc2 (1-415)) encoding the N-terminal region of the NFATc2 (amino acids 1-415), which contains the strong acidic transactivation domain (TAD-A) and the whole regulatory domain fused to the GAL4 DNA-binding domain. As expected from our previous results (18), expression of A238L strongly inhibits the function of NFAT transactivation domain (Fig. 7A). Reporter activity was not induced either in Jurkat-pcDNA or Jurkat-A238L by stimulation with PMA/Ion when the control GAL4-DNA binding domain was transfected (data not shown).

In the case of NFκB, and although the induced nuclear translocation of NFκB has been generally considered as the principal way to activate NFκB-dependent gene expression, an alternative mechanism of NFκB activation is emerging that involves the phosphorylation of the RelA/p65 transactivation subunit. To address this question, we used an approach similar to that described above for NFAT. Thus, a plasmid encoding the GAL4-p65 fusion protein, where the DNA-binding domain of GAL4 has been joined to the transactivation domain of RelA/p65 (28), was cotransfected with a GAL4-Luc reporter, allowing us to determine whether the viral protein down-regulates TNF-α gene expression by specifically targeting the transactivation domain of the RelA/p65 subunit of NFκB. Fig. 7B shows that in the presence of the A238L inhibitor, the ability of PMA/Ion to activate GAL4-p65 was strongly abrogated from 1 to 6 hours after stimulation.

As we mentioned before, overexpression of c-Jun by cotransfection of pRSV-c-Jun counteracted the inhibition of the TNF-α promoter activity observed in Jurkat-A238L. To assess whether c-Jun transactivation is modified in cells expressing the viral

protein, we cotransfected the plasmid encoding the GAL4-c-Jun fusion protein together with the GAL4-Luc plasmid in Jurkat-pcDNA and Jurkat-A238L. As shown in Fig. 7C, GAL4-c-Jun was poorly stimulated in Jurkat-A238L in comparison with the stimulation observed in Jurkat-pcDNA, suggesting that A238L targets also the transactivation domain of c-Jun.

CBP/p300 overexpress ion reverts the A238L-mediated inhibition of the pTNF(-120)luc promoter activity

CBP/p300 proteins play a critical role in the induction of TNF-α transcription by virus, T-cell receptor ligands, and LPS (6, 39, 40). CBP/p300 proteins function as coactivators for multiple transcription factors, including NFκB, NFAT and c-Jun. On the other hand, previous studies have shown that the composite TNF-α CRE/κ3 site, which binds ATF-2/Jun and NFAT proteins and is required for induction of the TNF-α gene by TCR engagement, virus infection, and calcium influx, is a CBP/p300-dependent element (12).

To characterize the involvement of transcriptional coactivators CBP/p300 in the TNF-α promoter activity down-regulation induced by the viral protein, we have cotransfected expression plasmids for CBP (pCMV-CBP), p300 (pRSV-p300), or both, together with the pTNF(-120)luc, into Jurkat-pcDNA or Jurkat-A238L cells. Sixteen hours after transfection the cells were cultured in the absence or presence of PMA/Ion during 4 h and assayed for luciferase activity. As shown in Fig. 8A, overexpression of CBP in A238L cells, rescued the activity of the promoter after stimulation with PMA/Ion in a dose-dependent manner, indicating the involvement of CBP in the modulation of TNF-α expression by the viral protein. We have performed a similar experiment using the pRSV-p300 construct that drives the expression of p300. As

shown in Fig. 8B, a high recovery of TNF-α promoter activity could be found in Jurkat-A238L in the presence of increasing doses of pRSV-p300, thus demonstrating the involvement of p300 in the mechanism of TNF-α inhibition by the viral protein. Next, we cotransfected the pTNF(-120)luc construct along with increasing amounts of pCMV-CBP and pRSV-p300 expression plasmids together. As shown in Fig. 8C, a complete reversion of the inhibition of TNF-α promoter activity was obtained. These results suggest that A238L-mediated TNF-α inhibition is accomplished by modulation of CBP/p300 transcriptional coactivators representing an accurate viral mechanism to evade the inflammatory immune response.

Discussion

In the current study we show that A238L specifically inhibits the TNF-α transcription by a mechanism that involves CBP/p300. The important role of A238L in controlling TNF-α synthesis was shown by the effect of virus deletion mutant both in infected Vero cells and porcine macrophages.

Despite the relevance of TNF-α in the control of viral infections, the role that this cytokine could play during ASFV infection *in vivo* (41, 42) or *in vitro* (33, 43), and the molecular mechanisms involved in the potential control of TNF-α by ASFV remained largely unknown. Previous reports have shown an up-regulation of TNF-α gene expression after infection with ASFV E75-infected macrophages (33). However, in stimulated macrophages, an inhibition of TNF-α production was observed from early times post-infection (43). These data could be expected if A238L, a protein synthesized from 6 hpi during the viral cycle, would inhibit NFkB/NFAT-dependent gene activation, although a direct effect of A238L on TNF-α gene expression was needed to explain these controversial results. Indeed, we and others have previously shown that A238L regulates the activity of NFAT and NF-κB (16, 17). In a recent work we have demonstrated that A238L down-regulates COX-2 transcription in a NFAT-dependent, but NF-κB-independent manner. The NF-κB site was not required for A238L inhibition and p65 NF-κB did not revert this inhibition, identifying NFAT as the target of A238L-mediated down-regulation of COX-2 promoter (18).

In this report, we provide compelling evidence that establishes that A238L efficiently controls TNF-α production during the ASFV infection of Vero cells or macrophages, since A238L deletion viruses induced an exacerbated TNF-α secretion. Moreover, ectopic expression of the viral protein also inhibits the TNF-α m-RNA expression after PMA/Ion stimulation in Jurkat cells.

The molecular mechanism by which A238L inhibits TNF-α has been investigated by examining the regulation of the TNF-α promoter activity by the viral protein. The region involved in the regulation of TNF-α transcription contains a CRE binding site, which binds ATF-2/Jun heterodimer and forms a composite element with the κ3 site (6, 34, 39). By using serial deletions constructs of TNF-α promoter, we found that the pTNF(-120)luc reporter plasmid, a deletion mutant of the promoter that only contains the composite CRE/κ3 site, was inhibited by A238L. In T cells activated by PMA/Ion treatment, a specific set of transcription factors is involved in the activation of the TNF-α enhancer. ATF-2/Jun is constitutively bound to this enhancer and NFAT binds to multiple sites in the promoter in response to ionophore stimulation (34). NFAT DNA-binding components can form Rel/NF-κB-like dimers on certain types of NFAT-binding DNA elements (14, 44, 45), such as the κ3 element of the TNF-α promoter, that binds NFAT dimers (14) as well as certain Rel-containing dimers (46).

In line with these results, we have found that the overexpression of NFAT, p65 and c-Jun activates TNF-α transcription and counteracts the inhibition of the TNF-α promoter activity mediated by A238L. This result not only corroborates that these transcription factors are involved in the control of TNF-α in Jurkat cells after PMA/Ion treatment, but also, and more importantly, that A238L interferes with all of them in the activation of TNF-α. In parallel with the nuclear accumulation of NF-κB and NFAT, activation of JNK1 by PMA/Ion in T cells results in the rapid phosphorylation of the constitutively bound c-Jun and ATF-2 heterodimer in the TNF-α promoter (47). In this regard, it is worth mentioning that neither down-regulation of c-Jun expression nor direct inhibition of the kinase activity of JNK1 could be found in Jurkat-A238L after

PMA/Ion stimulation, indicating that A238L is interfering with c-Jun-dependent TNF-α gene expression in a step down-stream phosphorylation by JNK1.

There are several mechanisms by which A238L could act to inhibit NFκB activation. The similarity between ankyrin repeats in A238L and IκB suggests that, like IκB, A238L may bind directly to NFκB. We have previously shown that p65 is co-precipitated with A238L from cell extracts infected with ASFV, suggesting that A238L is present in a complex with NFκB (17, 48) during the viral infection. Purified recombinant A238L protein added to nuclear extracts from stimulated cells inhibited binding of NFκB to target DNA sequences and displaced preformed NFκB complexes from DNA. Furthermore, we also showed in these experiments that recombinant A238L protein inhibited the formation of complexes containing p65/p50 heterodimers, rather than those containing p50 homodimers, as was expected, since p50 homodimers act to suppress NFκB-dependent transcription whereas p50/p65 heterodimers act as transcriptional transactivators.

In the present work we demonstrate that the expression of A238L in Jurkat cells does not inhibit the translocation of p65 to the nucleus after PMA/Ion stimulation, further supporting the hypothesis that A238L-mediated NFκB inhibition does not involves hijacking of p65 in the cytoplasm. In relation to this, it is known that, although a major step in NFκB activation is the removal of IκB from the NFκB/IκB complex allowing its nuclear translocation, a second level of regulation of NFκB exists that depends on phosphorylation of the transactivation domain (TAD) enhancing its transcriptional activity. Thus, p65 phosphorylation at its TAD is thought to enhance transcriptional competency by recruiting co-activator proteins such as CBP/p300 to NF-κB.

p300 is a member of a family of transcriptional coadaptor molecules with distinct functional domains, that have been shown to interact with several viral proteins such as the adenovirus protein E1A, simian virus 40 large T antigen (49) and herpes virus E6 and E7 (50). The consequence of this interaction on the biological effects on p300 function differs depending on the specific viral proteins and, although both adenovirus E1A and simian virus 40 large T antigen interact with p300 in overlapping locations, large T antigen inhibits, whereas E1A enhances, the phosphorylation of p300 (51).

Although the A238L-induced inhibition of the stimulus-induced transactivation of NFAT, p65 and c-Jun, or the effect on the recovery of the inhibition of the TNF(-120) luc promoter construct activity by overexpression of CBP/p300 described here could explain how A238L down-regulates TNF-α promoter activity, the molecular basis for the A238L-induced inhibition of TNF-α gene expression are still not clearly defined. CBP and p300 are essential for the optimal transcriptional activity of TNF-α and COX-2 (12, 52), two genes inhibited by A238L. It is interesting to speculate that a viral gene such as A238L, which inhibits the transactivation of NFAT, NFκB and c-Jun in response to PMA/Ion, may have evolved this level of flexibility to accomplish novel patterns of gene regulation to evade the host response. It is tempting to speculate that A238L would interact with CBP/p300 thus inhibiting the transactivation of factors which associate with them as NFAT, NFκB and c-Jun.

Thus, the full understanding of TNF-α gene regulation in ASFV-infected cells and in A238L-stably expressing human T cells, could potentially lead to novel therapeutic manipulations that interrupt the signaling cascade resulting in TNF-α gene transcription and subsequent protein production in conditions characterized by TNF-α-mediated immuno-pathologic effects. A detailed understanding of the mechanism by

which A238L inhibits TNF-α gene transcription in different cells also offers potential novel insights regarding the role of TNF-α in several inflammatory pathologies.

The analysis presented here indicate for the first time that A238L is a novel viral protein with a potent regulatory function on TNF-α that might be mediated by the control of CBP/p300 activity.

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FOOTNOTES

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(3) Abbreviations

The abbreviations used are: IKK, IκB kinase; COX-2, cyclooxygenase-2; Cn, calcineurin; TAD, transactivation domain; CsA, cyclosporin A; Ion, calcium ionophore; X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid); RLU, relative luciferase unit.

Figure Legends

FIGURE 1. TNF-α promoter activity and mRNA levels in ASFV-infected Vero cells. *A,* Western blot of Vero cells mock-infected (M) or infected with the Vero-adapted strain Ba71V wt or Ba71VΔA238L. At the indicated post-infection times (hpi), whole-cell extracts were prepared, subjected to SDS-PAGE (30 μg of protein sample), and detected by immunoblotting with an A238L specific antibody. A control of protein loading is included by β-actin blotting. *B,* Vero cells were transfected with the pTNF(–1311)luc plasmid (containing the full-length promoter sequence of human TNF-α gene fused to the firefly luciferase reporter gene) as described under *Materials and methods*. Sixteen hours after transfection the cells were mock-infected (Mock) or infected with Ba71V wt or Ba71VΔA238L. Whole-cell extracts were prepared at the indicated post-infection times and assayed for luciferase activity. Relative light units (RLU) per μg of protein from triplicate transfections (mean ± S.D.) are shown. *C,* RT-PCR analysis of infected Vero cells. Total RNA (1 μg) from Vero cells mock-infected (M) or infected with Ba71V wt or Ba71VΔA238L viruses was prepared at the indicated post-infection times and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) to measure TNF-α and A238L mRNA levels. A control using specific oligonucleotides for β-actin is also included to rule out differences in PCR amplification. Amplified DNA was separated on an agarose gel and stained with ethidium bromide.

FIGURE 2. TNF-α promoter activity, mRNA and protein levels in ASFV-infected macrophages. *A,* Characterization of the E70 A238L deletion mutant by Southern blot analysis of parental E70 virus (WT) and ΔA238L (Δ). Purified viral DNA digested with

Eco RI was electrophoresed, blotted, and hybridized with the indicated probes as described under *Material and methods. B,* RT-PCR analysis of infected porcine alveolar macrophages. Total RNA (1 μg) from macrophages mock-infected (M) or infected with E70 wt or E70ΔA238L virus was prepared at the indicated post-infection times and analyzed by RT-PCR to measure TNF-α and A238L mRNA levels. A control using specific oligonucleotides for porcine GAPDH is also included to rule out differences in PCR amplification. Amplified DNA was separated on an agarose gel and stained with ethidium bromide. *C,* Porcine alveolar macrophages were transfected with the pTNF(–1311)luc plasmid as described under *Materials and methods,* and mock-infected (Mock) or infected with the virulent isolate E70 wt or E70ΔA238L 4 h after transfection. Whole-cell extracts were prepared at the indicated post-infection times and assayed for luciferase activity. Relative light units (RLU) per μg of protein from triplicate transfections (mean ± S.D.) are shown. *D,* Porcine alveolar macrophages were infected with the virulent isolate E70 wt or E70ΔA238L mutant, supernatants were recovered at the indicated post-infection times and assayed in TNF-α ELISA as described under *Materials and methods.*

FIGURE 3. Analysis of TNF-α expression in stably expressing A238L Jurkat cells. *A,* Total RNA (1 μg) from Jurkat-pcDNA and Jurkat-A238L cells cultured in the absence (NS) or presence of 15 ng/ml of PMA plus 1 μM Ion (PMA+Ion) at the indicated times was analyzed by RT-PCR to measure TNF-α and A238L mRNA expression. A control using specific oligonucleotides for β-actin is also included to rule out differences in PCR amplification. Amplified DNA was separated on an agarose gel and stained with ethidium bromide. *B,* Jurkat-pcDNA or Jurkat-A238L cells were transiently transfected with the pTNF(–1311)luc TNF-α human promoter construct as described under

Materials and methods. Sixteen hours after transfection cells were cultured in the absence (open bars) or presence of 15 ng/ml of PMA plus1 μM of Ion (shaded bars) for 4 h and assayed for luciferase activity. Results from triplicate assays are shown in relative light units (RLU) per μg of protein (mean ± S.D.).

FIGURE 4. Effect of A238L upon the transcriptional activation of the TNF-α promoter. Jurkat-pcDNA (plain bars) or Jurkat-A238L (stripped bars) cells were transiently transfected with the indicated TNF-α promoter constructs as described under *Materials and methods*. Sixteen hours after transfection cells were cultured in the absence or presence of 15 ng/ml of PMA plus 1 μM of Ion for 4 h and assayed for luciferase activity. Results from triplicate assays are shown as bars in relative light units (RLU) per μg of protein (mean ± S.D.), corresponding to the series of 5'-truncations ranging from -1311 to –36 represented at the left-hand side of the figure. Cis-acting consensus sequences are represented by boxes. The extent of the 5'-truncations are shown with numbers indicating their length relative to the transcription start site. pTNF(–36)luc is also included to show the absence of luciferase activity after stimulation under our experimental conditions.

FIGURE 5. NFAT, p65 and c-Jun, but not c-Fos, participate in the transcriptional activation of the TNF-α gene promoter modulated by A238L. Jurkat-pcDNA (plain bars) or Jurkat-A238L (stripped bars) cells were transiently transfected with the pTNF(–120)luc reporter plasmid (a 5' deletion construct which preserves the CRE (-107 to –99 nt) and κ3 (-97 to –88 nt) sites) and with the indicated doses (from 0 to 500 ng of DNA per million cells) of four different expression plasmids: *A,* p1SH107c to overexpress the NFAT transcription factor, *B,* pCMV-p65 to overexpress the NFκB transcription factor,

C, pRSV-c-Jun, to overexpress the AP-1 family member c-Jun transcription factor, and *D,* pRSV-c-Fos to overexpress the AP-1 family member c-Fos transcription factor. Sixteen hours after transfection the cells were cultured in the absence (open bars) or presence of 15 ng/ml of PMA plus 1 μM Ion (shaded bars) during 4 h and assayed for luciferase activity. Results from triplicate assays are shown in relative light units (RLU) per μg of protein (mean ± S.D.).

FIGURE 6. A238L does not inhibit neither NFAT, NFκB-p65 nuclear translocation nor c-Jun activation. *A, s*ubcellular localization of NFAT in the presence or absence of A238L. Stably expressing A238L Vero cells (Vero-A238L) or control cells (Vero-pcDNA) were cultured in the absence (control) or presence of 15 ng/ml of PMA plus 1 μM Ion during 15, 30 or 90 min, or pre-incubated for 1 h with CsA and then stimulated with 15 ng/ml of PMA plus 1 μM Ion during 90 minutes (CsA + 90 min). Then the cells were labeled with an anti-NFAT antibody (green), and examined by confocal microscopy. The figure shows images corresponding to one of three independent experiments performed. *B,* cytosolic and nuclear extracts from Jurkat-pcDNA and Jurkat-A238L cells non-stimulated (NS), stimulated with 15 ng/ml of PMA plus 1 μM Ion (PMA+Ion) during 30 min (30') or 90 min (90') were prepared, subjected to SDS-PAGE (30 μg of cytosolic extract and the corresponding fraction of nuclear extract), and detected by immunoblotting with a NFκB-p65 specific antibody. A control of cellular fractions and protein loading is included by β-actin blotting. *C,* Whole cell extracts from Jurkat-pcDNA and Jurkat-A238L cells non-stimulated (NS), stimulated with 15 ng/ml of PMA plus 1 μM Ion (PMA+Ion) during 15 min (15'), 30 min (30') or 90 min (90') were prepared, subjected to SDS-PAGE (30 μg of protein), and detected by immunoblotting with a c-Jun specific antibody. *D,* whole cell extracts from 10^7

stably transfected Jurkat-pcDNA and Jurkat-A238L cells cultured in the absence or presence of 15 ng/ml of PMA plus 1 μM Ion during 30 minutes (30'), were incubated and immunoprecipitated with 1 μg of JNK1 specific antibody. In the left panel, immunoprecipitates were separated by SDS-PAGE, transferred to an immobilon membrane and revealed with the same JNK1 antibody. In the right panel, the immunoprecipitates were used in an in vitro kinase assay as described under *Materials and methods,* using purified GST-c-Jun as the substrate. Proteins were separated by SDS-12% PAGE and developed by autoradiography.

FIGURE 7. A238L inhibits transactivation mediated by NFAT, p65 and c-Jun. *A,* Jurkat-pcDNA and Jurkat-A238L cells were cotransfected with GAL4-NFAT (50 ng DNA/10^6 cells) and GAL4-luc (150 ng DNA/10^6 cells) and cultured with 15 ng/ml of PMA plus 1 μM Ion. *B,* Jurkat-pcDNA and Jurkat-A238L cells were cotransfected with GAL4-p65 (150 ng DNA/10^6 cells) and GAL4-luc (150 ng DNA/10^6 cells) and cultured with 15 ng/ml of PMA plus 1 μM Ion. *C,* Jurkat-pcDNA and Jurkat-A238L cells were cotransfected with GAL4-c-Jun (150 ng DNA/10^6 cells) and GAL4-luc (150 ng DNA/10^6 cells) and cultured with 15 ng/ml of PMA plus 1 μM Ion. In each case whole-cell extracts were prepared at the indicated post-stimulation times and luciferase activity was assayed. Relative light units (RLU) per μg of protein from triplicate transfections (mean ± S.D.) are shown.

FIGURE 8. CBP and p300 expression counteract the inhibition of TNF-α promoter activity induced by A238L. Jurkat-pcDNA (plain bars) or Jurkat-A238L (stripped bars) cells were transiently transfected with the pTNF(–120)luc reporter plasmid (a 5' deletion construct which preserves the CRE (-107 to –99 nt) and κ3 (-97 to –88 nt)

sites) and with the indicated doses (from 0 to 500 ng of DNA per 10^6 cells) of CREB Binding Protein (CBP) and p300 expression plasmids: *A,* pRC/RSV-CBP-HA to overexpress the CBP transcriptional coactivator. *B,* pCMVβ-p300-HA to overexpress the p300 transcriptional coactivator. *C,* pCMVβ-p300-HA and pRC/RSV-CBP-HA to overexpress both coactivators. Sixteen hours after transfection the cells were cultured in the absence (open bars) or presence of 15 ng/ml of PMA plus 1 μM Ion (shaded bars) during 4 h and assayed for luciferase activity. Results from triplicate assays are shown in relative light units (RLU) per μg of protein (mean ± S.D.).

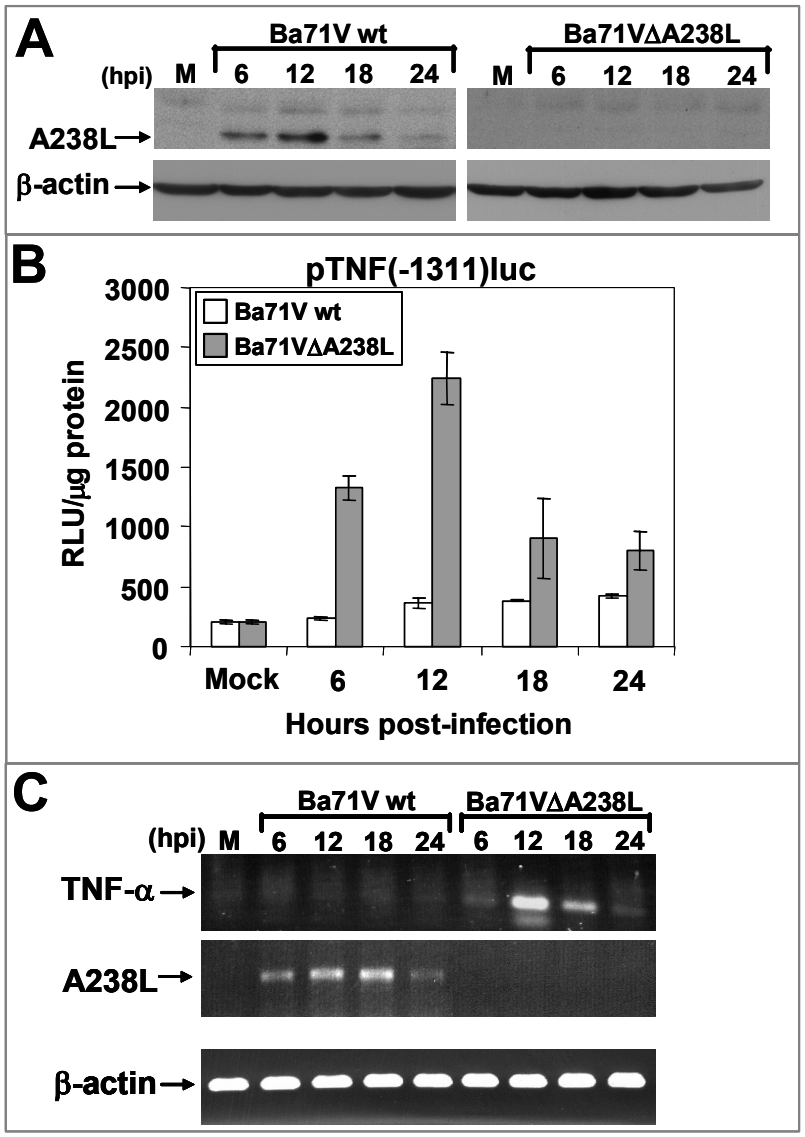
A
Ba71V wt
Ba71VΔA238L
(hpi) M 6 12 18 24 M 6 12 18 24
A238L
β-actin
B
pTNF(-1311)luc
Ba71V wt
Ba71VΔA238L
RLU/μg protein
3000
2500
2000
1500
1000
500
0
Mock 6 12 18 24
Hours post-infection
C
Ba71V wt
Ba71VΔA238L
(hpi) M 6 12 18 24 6 12 18 24
TNF-α
A238L
β-actin

Fig. 1

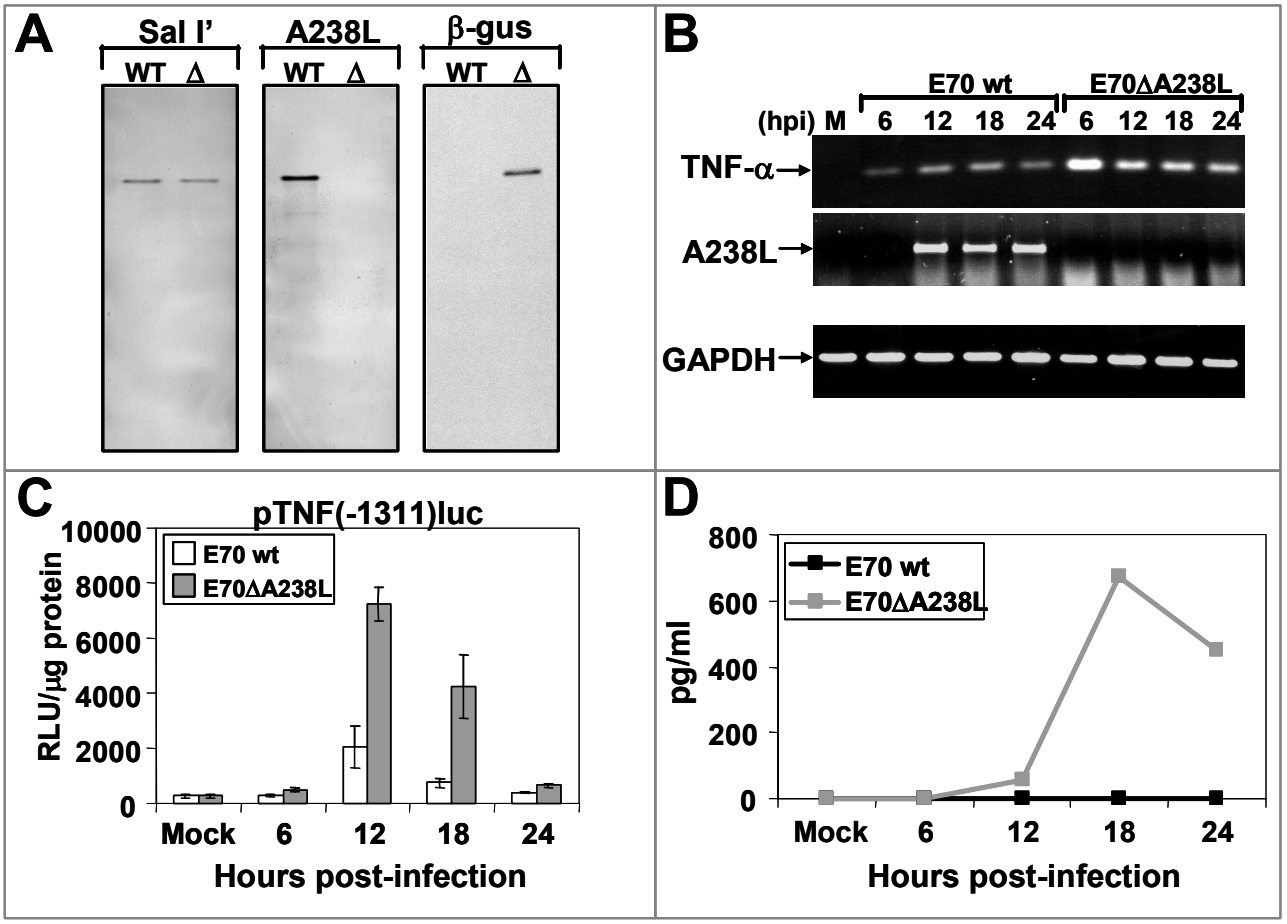
A
Sal I'
A238L
β-gus
WT Δ
WT Δ
WT Δ
B
E70 wt
E70ΔA238L
(hpi) M 6 12 18 24 6 12 18 24
TNF-α
A238L
GAPDH
C
pTNF(-1311)luc
E70 wt
E70ΔA238L
RLU/μg protein
10000
8000
6000
4000
2000
0
Mock 6 12 18 24
Hours post-infection
D
E70 wt
E70ΔA238L
pg/ml
800
600
400
200
0
Mock 6 12 18 24
Hours post-infection

Fig. 2

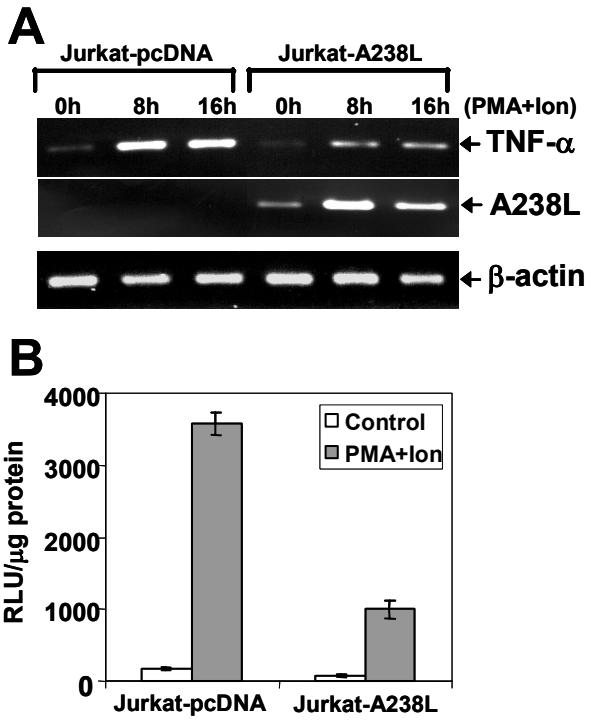
A
Jurkat-pcDNA
Jurkat-A238L
0h
8h
16h
0h
8h
16h
(PMA+Ion)
TNF-α
A238L
β-actin
B
4000
3000
2000
1000
0
RLU/μg protein
Control
PMA+Ion
Jurkat-pcDNA
Jurkat-A238L

Fig. 3

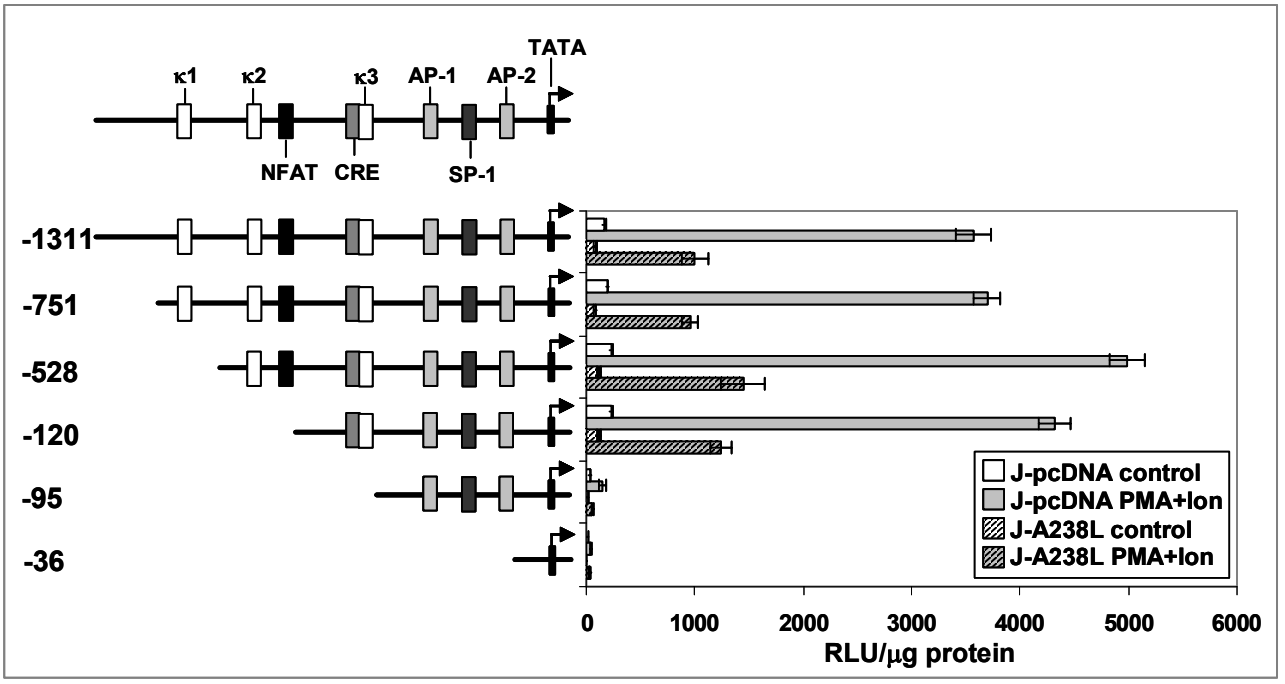
TATA
κ1
κ2
κ3
AP-1
AP-2
NFAT
CRE
SP-1
-1311
-751
-528
-120
-95
-36
J-pcDNA control
J-pcDNA PMA+Ion
J-A238L control
J-A238L PMA+Ion
0
1000
2000
3000
4000
5000
6000
RLU/μg protein

Fig. 4

pTNF(-120) luc

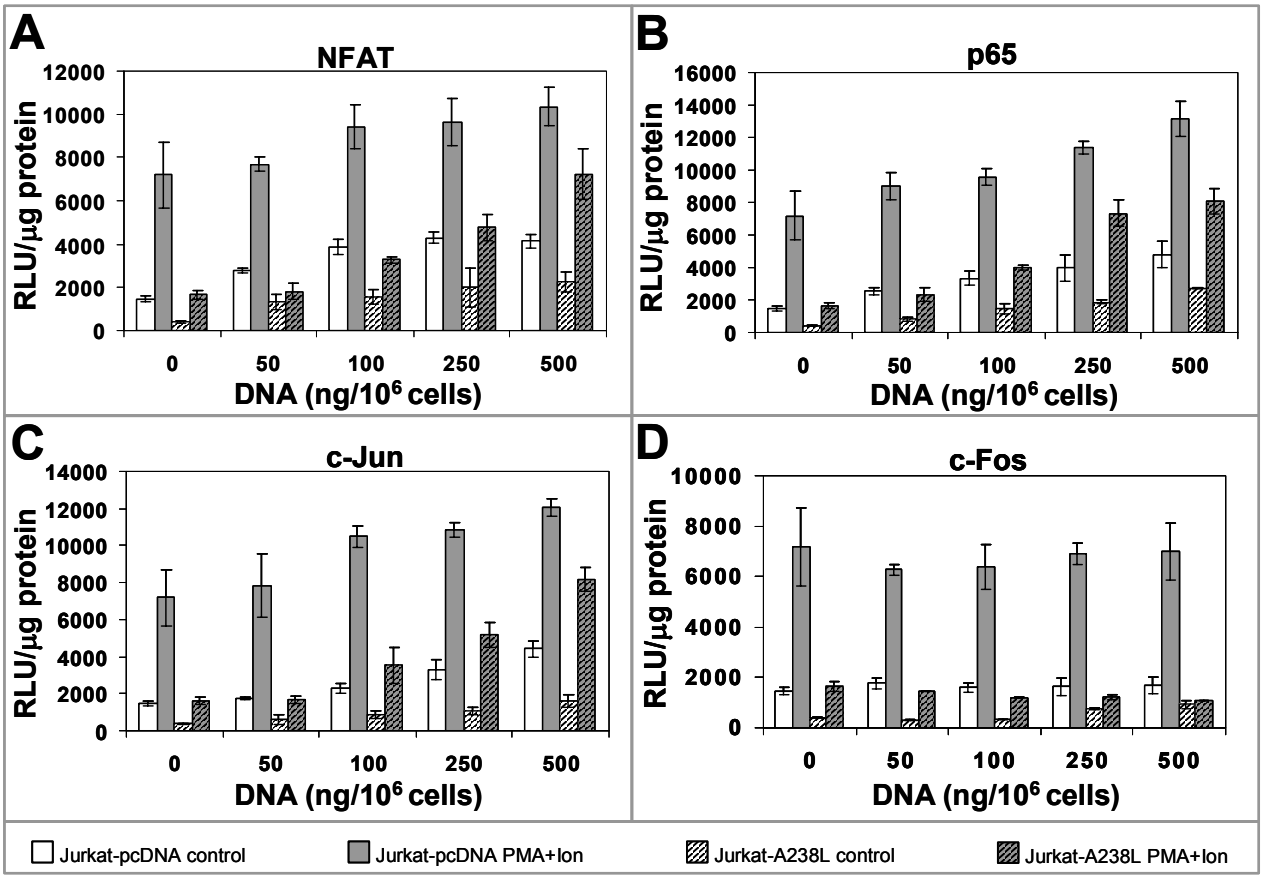

Fig. 5

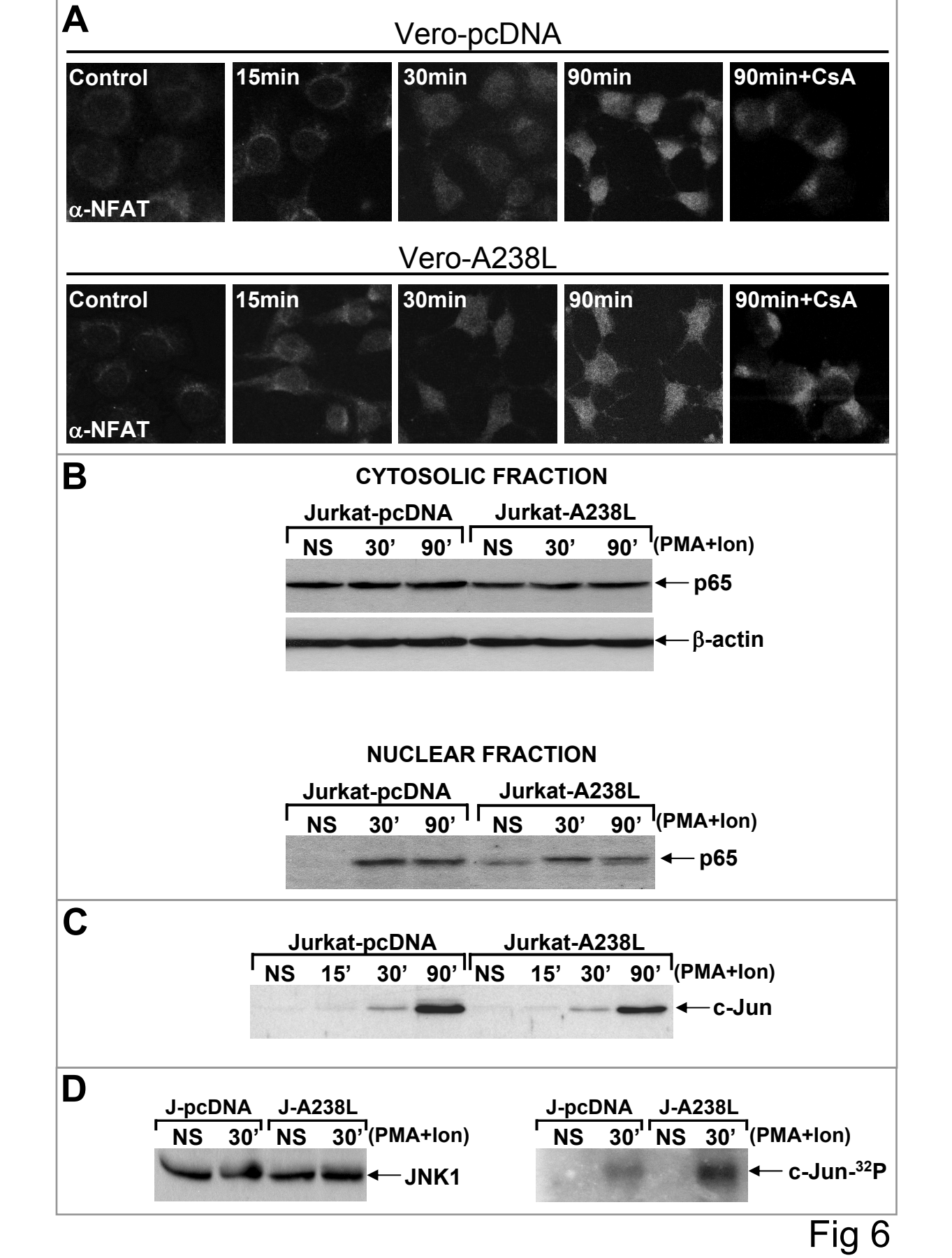
A
Vero-pcDNA
Control
15min
30min
90min
90min+CsA
α-NFAT
Vero-A238L
Control
15min
30min
90min
90min+CsA
α-NFAT
B
CYTOSOLIC FRACTION
Jurkat-pcDNA
Jurkat-A238L
NS
30'
90'
NS
30'
90'
(PMA+Ion)
p65
β-actin
NUCLEAR FRACTION
Jurkat-pcDNA
Jurkat-A238L
NS
30'
90'
NS
30'
90'
(PMA+Ion)
p65
C
Jurkat-pcDNA
Jurkat-A238L
NS
15'
30'
90'
NS
15'
30'
90'
(PMA+Ion)
c-Jun
D
J-pcDNA
J-A238L
NS
30'
NS
30'
(PMA+Ion)
JNK1
J-pcDNA
J-A238L
NS
30'
NS
30'
(PMA+Ion)
c-Jun-32P

Fig 6

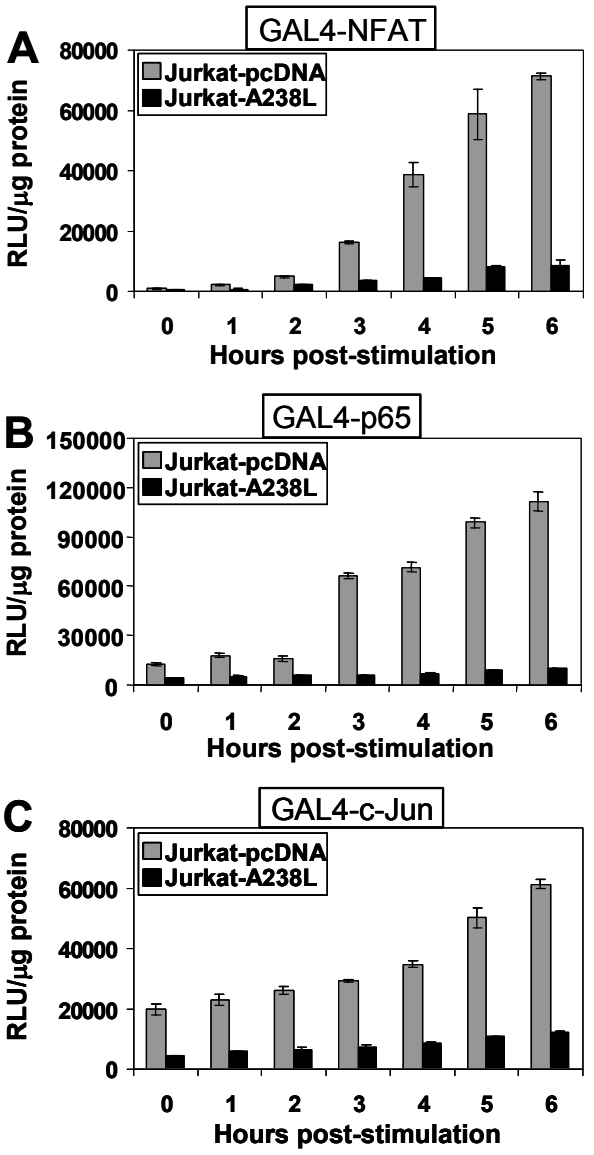
A
GAL4-NFAT
RLU/µg protein
80000
60000
40000
20000
0
Jurkat-pcDNA
Jurkat-A238L
0 1 2 3 4 5 6
Hours post-stimulation
B
GAL4-p65
RLU/µg protein
150000
120000
90000
60000
30000
0
Jurkat-pcDNA
Jurkat-A238L
0 1 2 3 4 5 6
Hours post-stimulation
C
GAL4-c-Jun
RLU/µg protein
80000
60000
40000
20000
0
Jurkat-pcDNA
Jurkat-A238L
0 1 2 3 4 5 6
Hours post-stimulation

Fig. 7

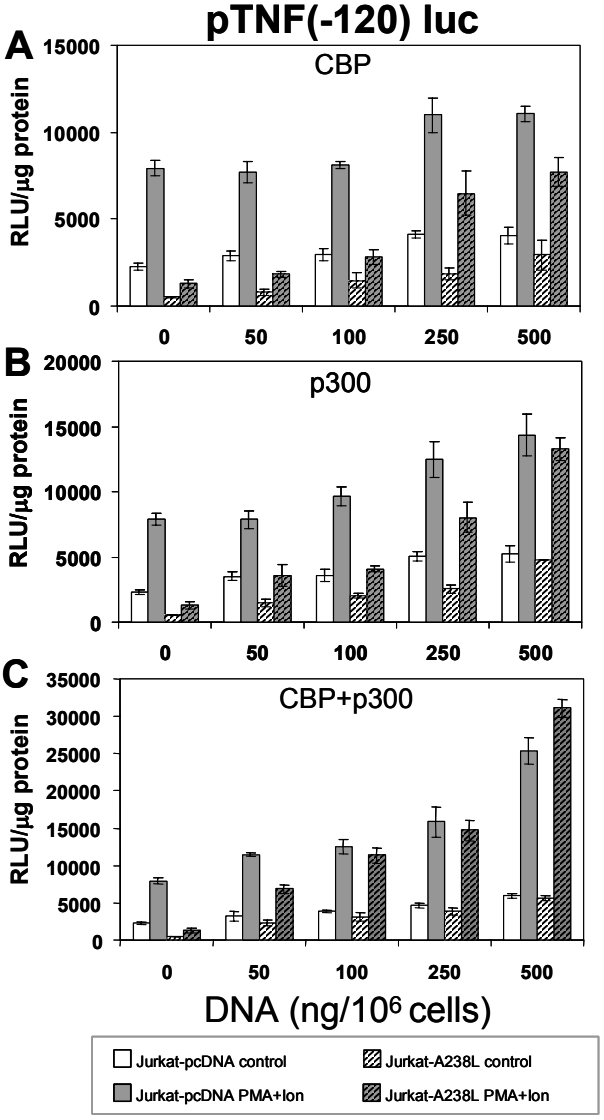
pTNF(-120) luc
A
CBP
B
p300
C
CBP+p300
RLU/µg protein
15000
10000
5000
0
20000
35000
30000
25000
0
50
100
250
500
DNA (ng/10⁶ cells)
Jurkat-pcDNA control
Jurkat-A238L control
Jurkat-pcDNA PMA+Ion
Jurkat-A238L PMA+Ion

Fig. 8