

# The Poxvirus Protein A52R Targets Toll-like Receptor Signaling Complexes to Suppress Host Defense

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## Abstract

Toll-like receptors (TLRs) are crucial in the innate immune response to pathogens, in that they recognize and respond to pathogen associated molecular patterns, which leads to activation of intracellular signaling pathways and altered gene expression. Vaccinia virus (VV), the poxvirus used to vaccinate against smallpox, encodes proteins that antagonize important components of host antiviral defense. Here we show that the VV protein A52R blocks the activation of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) by multiple TLRs, including TLR3, a recently identified receptor for viral RNA. A52R associates with both interleukin 1 receptor-associated kinase 2 (IRAK2) and tumor necrosis factor receptor-associated factor 6 (TRAF6), two key proteins important in TLR signal transduction. Further, A52R could disrupt signaling complexes containing these proteins. A virus deletion mutant lacking the A52R gene was attenuated compared with wild-type and revertant controls in a murine intranasal model of infection. This study reveals a novel mechanism used by VV to suppress the host immunity. We demonstrate viral disabling of TLRs, providing further evidence for an important role for this family of receptors in the antiviral response.

Key words: nuclear factor  $\kappa$ B • signal transduction • vaccinia virus • immunomodulation • Toll-like receptor

## Introduction

Members of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR)\* superfamily are key mediators in innate and adaptive immunity (1). The superfamily is defined by the presence of a cytosolic motif termed the Toll/IL-1 receptor (TIR) domain (1). The family includes receptors for the proinflammatory cytokines IL-1 and IL-18 as well as the TLR members, which participate in the recognition of pathogens by responding to pathogen-associated molecular

patterns and activating signaling pathways leading to altered gene expression (1, 2). 10 mammalian TLRs have been identified to date. TLR4, TLR5, and TLR9 are essential in the respective recognition of LPS, bacterial flagellin, and unmethylated CpG motifs that are present in bacterial DNA (3–6). TLR2 recognizes bacterial lipoproteins and other Gram-positive molecular patterns, but only when present as a heterodimer in combination with either TLR1 or TLR6 (7–12).

TLRs have also been implicated in sensing viral infections (13–17). Most notably, TLR3 is activated in response to poly(I:C), a synthetic double-stranded RNA (dsRNA) mimic of viral dsRNA, which is a molecular pattern produced by most viruses (15). Poly(I:C) activation of cells via TLR3 led to the activation of the transcription factor nuclear factor (NF)- $\kappa$ B and the production of type I interferons, which are important in antiviral innate immunity (15).

A. Bowie and L. O'Neill contributed equally to the work.

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\*Abbreviations used in this paper: dsRNA, double-stranded RNA; IP, immunoprecipitation; IRAK, IL-1 receptor-associated kinase; Mal, MyD88 adaptor-like; NF, nuclear factor; TAB, TAK1 binding protein; TAK1, TGF- $\beta$ -activated kinase 1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRAF, TNF receptor associated factor; VV, vaccinia virus.

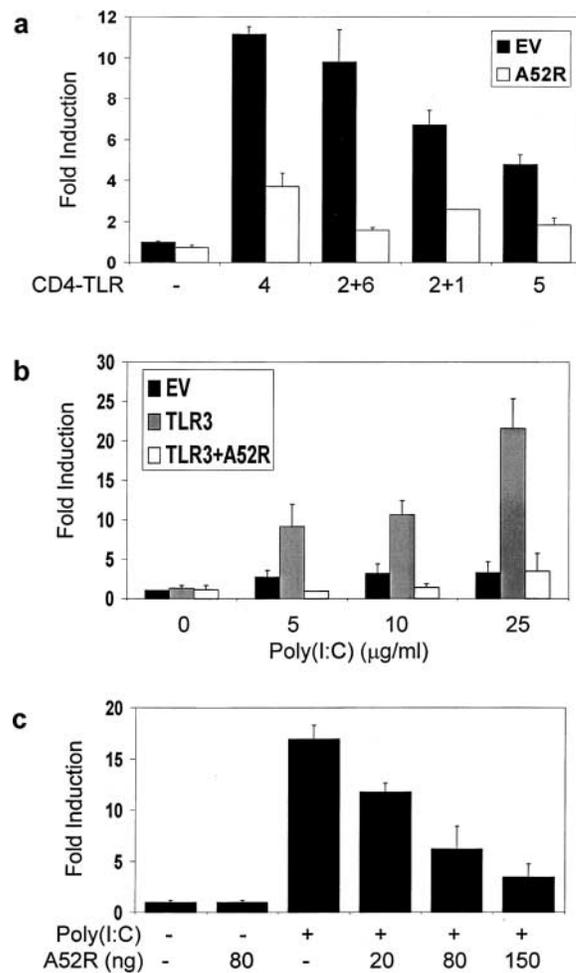
Stimulation of IL-1R/TLR family members with the appropriate ligands leads to activation of NF- $\kappa$ B and also the mitogen-activated protein kinases p38, JNK, and p42/44. The activation of NF- $\kappa$ B by IL-1, IL-18, TLR2, TLR7, and TLR9 is absolutely dependent on the cytoplasmic TIR domain-containing protein MyD88 (16, 18–20), which is recruited to receptor TIR domains (21–23). However, both TLR4 and TLR3 are able to activate NF- $\kappa$ B, by both a MyD88-dependent and MyD88-independent pathway (15, 24). For TLR4, the MyD88-independent pathway may be mediated by MyD88 adaptor-like (Mal, also known as TIRAP), as it has been demonstrated that Mal is recruited to TLR4, and is involved in NF- $\kappa$ B activation (25, 26). Activation of NF- $\kappa$ B by the MyD88-dependent pathway can proceed via recruitment of IL-1 receptor-associated kinase (IRAK) and/or IRAK2, while Mal can recruit IRAK2 (25). IRAK or IRAK2 activation in turn leads to recruitment of TNF receptor-associated factor 6 (TRAF6). TRAF6 is required for the ubiquitination and activation of TGF- $\beta$ -activated kinase 1 (TAK1), which, in complex with TAK1 binding protein 1 (TAB1), phosphorylates I $\kappa$ B kinase (IKK) leading to NF- $\kappa$ B activation (27).

Vaccinia virus (VV), the vaccine used to eradicate smallpox, encodes many immunomodulatory proteins to evade the host immune response (28). Previously we demonstrated that VV protein A52R down-regulated the activation of NF- $\kappa$ B by the IL-1R/TLR superfamily members IL-1R, IL-18R, and TLR4 (29). Here we show that A52R inhibits multiple TLR pathways to NF- $\kappa$ B, including TLR3, and provide evidence to suggest the mechanism of action. Further we show that A52R contributes to VV virulence in vivo. Thus, we demonstrate viral targeting of TLR signaling molecules, and reveal a novel mechanism used by VV to suppress the host immune response.

## Materials and Methods

**Expression Plasmids.** Chimeric TLR receptors CD4-TLR1, CD4-TLR2, CD4-TLR4, CD4-TLR5, and CD4-TLR6 composed of the extracellular domain of CD4 fused to the transmembrane domain and cytosolic tail of the TLR were a gift from R. Medzhitov (Yale University, New Haven, CT). TLR3 was a provided by K. Fitzgerald and D. Golenbock (University of Massachusetts Medical School, Worcester, MA). AU1-MyD88, Myc-IRAK2, and Myc-kIRAK2 expression vectors were a gift from M. Muzio, Mario Negri Institute, Milano, Italy (23). IRAK, Flag-TRAF6, Flag-TRAF6 domain (amino acids 289–522), and Flag-TRAF2 expression plasmids were provided by Tularik Inc. (San Francisco, CA). Flag-TAK-1 and HA-TAB-1 expression plasmids were a gift from H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). Construction of A52R, Flag-A52R, and HA-Mal expression plasmids was described previously (25, 29).

**Antibodies.** Polyclonal antibodies were raised against a purified, bacterially expressed glutathione S-transferase (GST) fusion of A52R, encoded by a plasmid synthesized by inserting full-length A52R downstream of GST in the bacterial expression vector GEX4T2. Other antibodies used were Anti-flag M2 monoclonal antibody, anti-flag M2 conjugated agarose, anti-myc

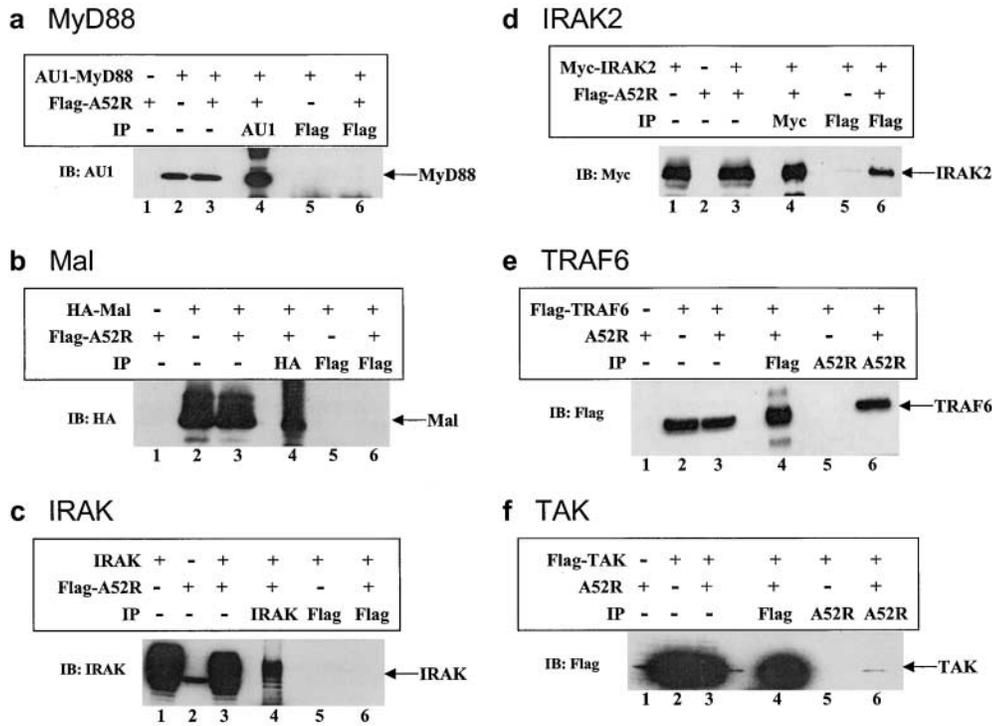


**Figure 1.** A52R inhibits NF- $\kappa$ B activation by multiple TLRs. (A) 293 cells were transfected with constitutively active CD4-TLRs (50 ng TLR4 or TLR5, or 25 ng each of TLR2 and TLR6 or TLR2 and TLR1) in the presence of 80 ng empty vector (EV) or plasmid encoding A52R, together with NF- $\kappa$ B reporter plasmid. (B) Cells were transfected with empty vector (EV), 0.5 ng TLR3, or 0.5 ng TLR3 plus 150 ng A52R. 6 h before harvesting cells were stimulated with 0–25  $\mu$ g/ml poly(I:C). (C) Cells were transfected with 0.5 ng TLR3 and stimulated with 25  $\mu$ g/ml poly(I:C) where indicated (+), in the presence of increasing amounts of A52R (20–150 ng). In all cases, luciferase activity was measured after 24 h.

monoclonal antibody clone 9E10 (all from Sigma-Aldrich), anti-AU1 monoclonal antibody (BabCO), anti-HA polyclonal antibody (Y-11), and anti-TRAF6 (H-274; both from Santa Cruz Biotechnology, Inc.). Anti-IRAK antibody was a gift from K. Ray (GlaxoSmithKline).

**NF- $\kappa$ B Reporter Gene Assay.** HEK 293 cells ( $2 \times 10^4$  cells per well) were seeded into 96-well plates and transfected the next day with expression vectors,  $\kappa$ B-luciferase reporter gene, and *Renilla*-luciferase internal control as described previously (25). The total amount of DNA per transfection was kept constant at 220 ng by addition of pcDNA3.1 (Stratagene). After 24 h, reporter gene activity was measured (25). Data are expressed as mean fold induction  $\pm$  SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

**Immunoprecipitation and Immunoblotting.** HEK 293T cells were seeded into 100-mm dishes ( $1.5 \times 10^6$ ) 24 h before transfection.



**Figure 2.** A52R immunoprecipitates with both IRAK2 and TRAF6. 293T cells were transfected with 4  $\mu$ g A52R (either flag-tagged or untagged, as indicated) plus 4  $\mu$ g of AU1-MyD88 (A), HA-Mal (B), IRAK (C), Myc-IRAK2 (D), Flag-TRAF6 (E), or Flag-TAK-1 (F). After 24 h lysates were subject to immunoprecipitation, and subsequent SDS-PAGE and immunoblotting with the antibodies indicated. Lysates were analyzed to ensure appropriate and equivalent expression of the proteins (lanes 1–3). To assess the efficiency of immunoprecipitation the immune complex (1/7th total lysate) was blotted with the same antibody used for immunoprecipitation (lane 4). Single transfection of each construct was also performed to act as a specificity control for the immunoprecipitating antibody (lane 5). The remainder of the immune complex was probed for the presence of associated protein (lane 6). The appearance of a band in lane 6 indicates an interaction. Representative of two (F) or three (A–E) independent experiments.

Transfections were performed using FuGENE 6 (Roche) according to manufacturer's instructions. For coimmunoprecipitations, 4  $\mu$ g of each construct was transfected. Where only one construct was expressed the total amount of DNA (8  $\mu$ g) was kept constant by supplementation with vector DNA. For competition experiments where the effect of increasing A52R expression on complex formation between two signaling molecules was examined, 2  $\mu$ g of each signaling molecule expression plasmid was transfected in the presence of increasing amounts of A52R expression plasmid. The total amount of DNA was kept constant in each sample using empty vector. In all cases, cells were harvested 24 h after transfection in 750  $\mu$ l of lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 containing 1 mM PMSF and protease inhibitor cocktail [Sigma-Aldrich], and 1 mM sodium orthovanadate). For immunoprecipitation, the indicated antibodies were precoupled to either protein A sepharose or protein G sepharose for 1 h at 4°C, washed, and then incubated with the cell lysates for 2 h at 4°C. The immune complexes were washed twice with lysis buffer and once with lysis buffer without NP-40 and glycerol. Associated proteins were eluted from the beads by boiling in 35  $\mu$ l sample buffer and fractionated by SDS PAGE. 30  $\mu$ l of the immune complex was immunoblotted for co-precipitating protein and the remaining 5  $\mu$ l was blotted directly for the protein directly recognized by the immunoprecipitating antibody. For immunoblotting, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence (Amersham Biosciences).

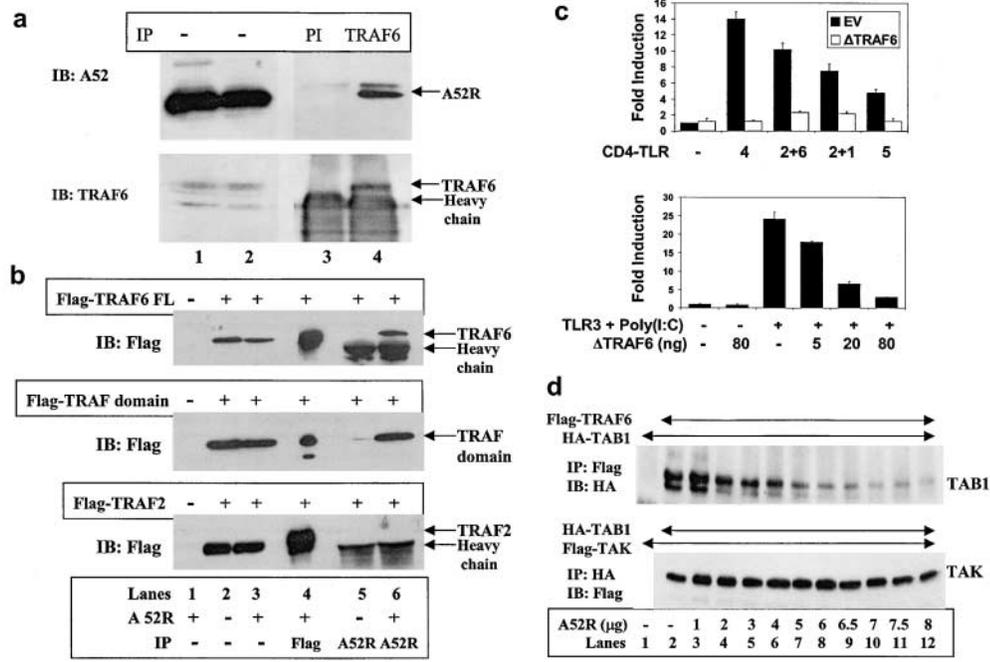
**Recombinant VV Viruses.** A VV mutant lacking 95.2% of the A52R gene (v $\Delta$ A52R) was constructed by transient dominant selection (30). A plaque purified wild-type virus (vA52R-wt) and a revertant virus (vA52R-rev) in which the A52R gene was reinserted at its natural locus were also isolated. The virulence of the viruses was investigated in a mouse intranasal model. Female, 6-wk-old Balb/c mice were anaesthetized and inoculated with 10<sup>4</sup> PFU of VV in 20  $\mu$ l of phosphate-buffered saline. A control group

was mock infected with PBS. Each day the weights of the animals and signs of illness were measured as described previously (31).

## Results

**A52R Inhibits NF- $\kappa$ B Activation by Multiple TLRs.** We have previously shown that A52R blocks the activation of NF- $\kappa$ B by IL-1, IL-18, and TLR4, with little inhibitory effect on the activation of NF- $\kappa$ B by TNF (29). Given the emerging role of TLRs in antiviral responses, we tested the effect of A52R on NF- $\kappa$ B activation by other TLRs. Chimeric versions of the TLRs, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of a human TLR render TLRs constitutively active, and have proved useful in probing TLR signaling pathways (5, 11, 32). Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), while others signal as heterodimers (TLR1, TLR2, and TLR6; references 5 and 11). Using these chimeras TLR signaling can be examined in the absence of exogenous activator. Induction of an NF- $\kappa$ B-dependent reporter gene by overexpression of either CD4-TLR4 or CD4-TLR5, or a combination of either CD4-TLR2 and 6, or CD4-TLR2 and 1 in HEK293 cells was inhibited in all cases by coexpression of A52R (Fig. 1 A).

DsRNA is a molecular pattern associated with viral infection, and TLR3 has been shown to sensitize cells to activation by polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue (15). Therefore, we also tested the effect of A52R on TLR3-dependent NF- $\kappa$ B activation induced by poly(I:C). Transfection of HEK293 cells with TLR3 led to strong dose-dependent activation of NF- $\kappa$ B by poly(I:C), which was not seen in the absence of TLR3 (Fig. 1 B). This



**Figure 3.** Analysis of the interaction of A52R with TRAF6. (A) A52R immunoprecipitates with endogenous TRAF6. Lysates from 293T cells transfected with A52R were immunoprecipitated with either preimmune serum (PI, lane 3) or anti-TRAF6 serum (lane 4), followed by immunoblotting with either A52R (top panel) or TRAF6 (bottom panel) antibody. Direct immunoblot of cell lysates confirmed equal expression of A52R and TRAF6 (lanes 1 and 2). (B) A52R immunoprecipitates with the TRAF6 TRAF domain, but not with TRAF2. 293T cells were transfected with A52R plus TRAF6 (top panel), ΔTRAF6 (residues 289–522 consisting of the TRAF domain of TRAF6, middle panel), or TRAF2 (bottom panel). Lysates were prepared and immunoprecipitated with A52R or Flag antibody as indicated, before immunoblot with Flag antibody. (C) Inhibition

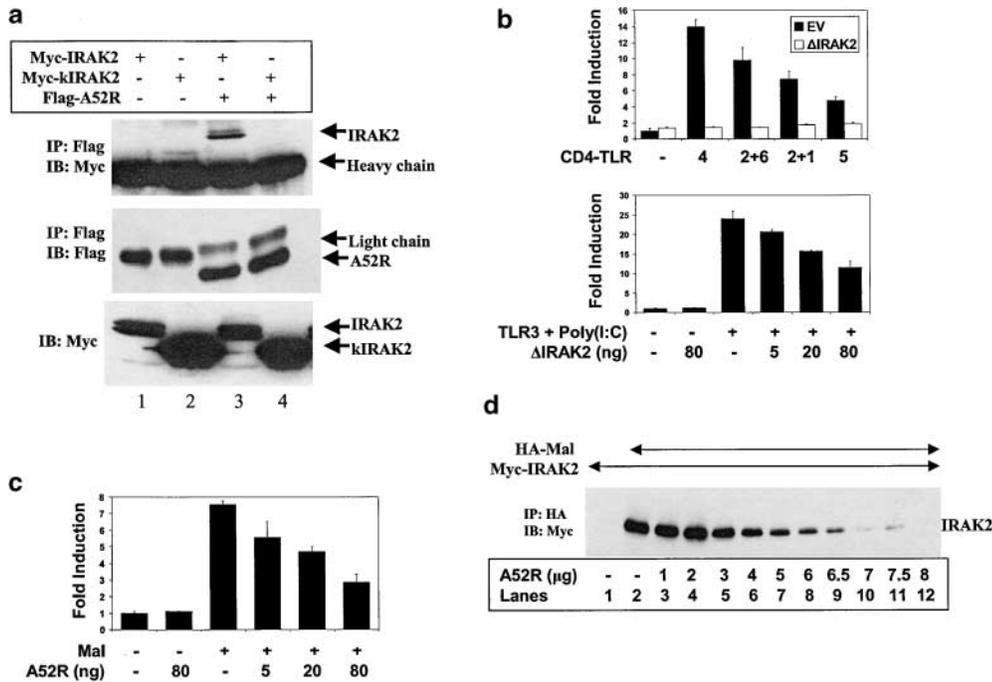
of TLR-induced NF-κB activation by ΔTRAF6. 293 cells were transfected with constitutively active CD4-TLRs as in Fig. 1, in the presence of 80 ng empty vector (EV) or plasmid encoding ΔTRAF6, together with NF-κB reporter plasmid (top panel). In the bottom panel, 293 cells were transfected with 0.5 ng TLR3 and stimulated with 25 μg/ml poly(I:C) where indicated (+). Luciferase activity was measured after 24 h. (D) A52R causes the dissociation of a TRAF6-TAB1-containing signaling complex but has no effect on a TAB1-TAK complex. A52R was cotransfected with either Flag-TRAF6 and HA-TAB1 (top panel) or HA-TAB1 and Flag-TAK1 (bottom r panel). Lysates were prepared and immunoprecipitated and immunoblotted as indicated. A52R can be seen to interfere with the TRAF6/TAB2 complex.

TLR3-dependent induction of NF-κB was completely blocked by A52R (Fig. 1 B) in a dose-dependent manner (Fig. 1 C). Thus, A52R is a global inhibitor of signaling by the TLR family, with TLR3 being particularly sensitive.

**A52R Interacts with TRAF6 and IRAK2.** The activation of NF-κB by different TLRs is mediated by a common set of signaling molecules (1). The ability of A52R to inhibit NF-κB activation by multiple TLRs suggested that its effects might be due to its interaction with a molecule whose function is critical to signaling by all TLRs. Thus, the ability of A52R to associate with characterized mediators of signaling of the TLR family was examined in coimmunoprecipitation (coIP) experiments. No complex formation was detected when A52R was coexpressed with MyD88 (Fig. 2 A), Mal (Fig. 2 B), or IRAK (Fig. 2 C), as indicated by the lack of a band in lane 6 in each case. Under the same conditions complexes were detected between MyD88 and IRAK2, Mal and IRAK2, and IRAK and TRAF6 (unpublished data) as has been demonstrated previously (23, 25, 33). However, upon expression of A52R with IRAK2 a clear complex of A52R with IRAK2 was immunoprecipitated, using antibody to either A52R (Fig. 2 D, lane 6), or IRAK2 (unpublished data). The next signaling mediator downstream of the IRAK family is TRAF6 (33). Coexpression of A52R with TRAF6 resulted in the formation of a complex with high stoichiometry, as detected by IP with an antibody to either A52R (Fig. 2 E, lane 6), or TRAF6 (unpublished data). TRAF6 is responsible for activating TAK1 that

forms a complex with its two coactivators TAB1 and TAB2 (27). A52R was coexpressed with either TAK1 or TAB1 to determine if it associates with either of these downstream mediators of TRAF6 signaling. A weak but reproducible interaction was detected between A52R and TAK1 (Fig. 2 F, lane 6). No interaction was detectable between A52R and TAB1 (unpublished data). These results indicate that A52R is capable of interacting with both IRAK2 and TRAF6. The interaction with TAK1 is likely to occur via endogenous TRAF6.

**A52R Associates with the TRAF6 TRAF Domain.** We next examined the specificity and functional consequences of the interaction of A52R with TRAF6-containing complexes. Fig. 3 A shows that A52R could be immunoprecipitated with endogenous TRAF6 (Fig. 3 A, top panel, compare lanes 3 and 4). To determine the regions in TRAF6 responsible for interacting with A52R, truncated versions of TRAF6 were coexpressed with A52R and tested for their ability to interact by IP. A truncated version of TRAF6 composed of just the TRAF domain was able to interact with A52R to the same extent as the full length TRAF6 (Fig. 3 B, lanes 5 and 6, compare top and middle panels). TRAF2 is a TRAF family member with a role in TNF signaling (34). A52R has only minimal inhibitory effect on the activation of NF-κB by TNFR (29) and consistent with this, no interaction between A52R and TRAF2 was detected (Fig. 3 B, bottom panel). A role for TRAF6 in the TLR pathways inhibited by A52R was confirmed by testing the effect of



**Figure 4.** Role of IRAK2 in inhibition of TLR-induced NF- $\kappa$ B activation by A52R. (A) A52R requires the IRAK2 death domain for association. 293T cells were cotransfected with A52R and either IRAK2, or kIRAK2 (lacking the death domain and containing residues 97–590). Lysates were immunoprecipitated and immunoblotted as indicated. The band in lane 3, top panel, indicates interaction with full-length IRAK2. (B) Inhibition of TLR-induced NF- $\kappa$ B activation by kIRAK2. 293 cells were transfected with constitutively active CD4-TLRs, as in Fig. 1, in the presence of 80 ng empty vector (EV) or plasmid encoding kIRAK2 ( $\Delta$ IRAK2), together with NF- $\kappa$ B reporter plasmid (top panel). In the bottom panel, 293 cells were transfected with 0.5 ng TLR3 and stimulated with 25  $\mu$ g/ml poly(I:C) where indicated (+). Luciferase activity was measured after 24 h. (C)

A52R inhibits the activation of NF- $\kappa$ B by Mal. 293 cells were transfected with 10 ng Mal where indicated (+), A52R, and NF- $\kappa$ B reporter plasmid. Luciferase activity was measured after 24 h. (D) A52R causes the dissociation of a Mal-IRAK2-containing complex. A52R was cotransfected with IRAK2 and Mal. The amount of IRAK2-Mal complex formed was assessed by immunoprecipitation using anti-HA antibody, followed by immunoblotting with anti-myc antibody. A52R can be seen to interfere with the Mal/IRAK2 complex.

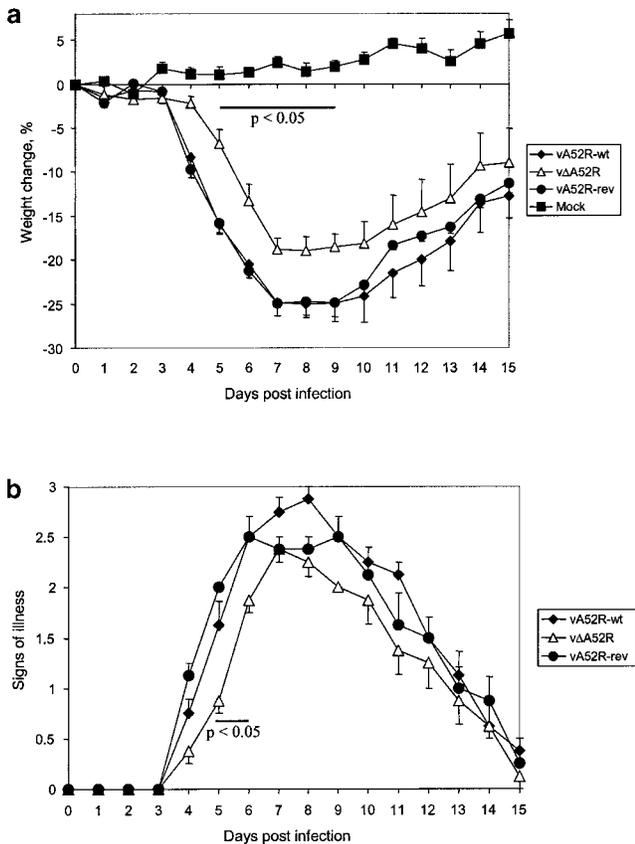
dominant negative TRAF6 (containing only the TRAF domain): Fig. 3 C shows that each CD4-TLR combination was inhibited (top panel), while 80 ng of plasmid encoding for dominant negative TRAF6 abolished the stimulatory effect of poly(I:C) (bottom panel). This is consistent with previous published work showing inhibition of TLR3-dependent NF- $\kappa$ B activation by dominant negative TRAF6 (15).

**A52R Disrupts a TRAF6-TAB1-containing Complex.** To test whether the ability of A52R to target TRAF6 could explain inhibition of TLR-induced NF- $\kappa$ B activation, the effect of A52R on the ability of TRAF6 to form active signaling complexes necessary for the activation of NF- $\kappa$ B was assessed. As an example of a TRAF6 complex important for NF- $\kappa$ B activation, we looked at the effect of A52R on a complex containing TRAF6 and TAB1 (27). We detected a TRAF6-TAB1-containing complex by IP (Fig. 3 D, top panel), and as the expression of A52R increased, the amount of TAB1 capable of coIP with TRAF6 decreased steadily (Fig. 3 D, top panel). This effect was specific to TRAF6 because the expression of increasing levels of A52R had no effect on the formation of a TAB1-TAK1 complex (Fig. 3 D, bottom panel).

**A52R Disrupts IRAK2-dependent Signaling.** Fig. 2 showed that A52R could also interact with IRAK2-containing complexes. We therefore examined the specificity and functional consequences of this interaction. The death domain of IRAK2 was required for association with A52R, as a truncated version of IRAK2 termed kIRAK2, which lacks the death domain, was unable to interact with A52R (Fig. 4 A, top panel, compare lanes 3 and 4). Similar to

TRAF6, there was a correlation between inhibition of TLR-induced NF- $\kappa$ B activation by A52R, and a role for IRAK2 in these pathways: Fig. 4 B shows that each CD4-TLR induced signal that was sensitive to A52R was also blocked by dominant negative IRAK2 (top panel). Further, we obtained the first evidence that IRAK2 has a role in TLR3-dependent poly(I:C)-induced NF- $\kappa$ B activation, as dominant negative IRAK2 dose dependently inhibited this signal (Fig. 4 B, bottom panel). Thus, IRAK2 has a wide-ranging role in many TLR pathways to NF- $\kappa$ B activation, providing a further rationale for the inhibitory effect of A52R on TLR signaling.

The activation of NF- $\kappa$ B by Mal requires IRAK2 (25). Given that IRAK2 is a target for A52R, the effect of A52R on the ability of Mal to activate NF- $\kappa$ B was examined. Overexpression of Mal activated NF- $\kappa$ B, and this activation was inhibited by the coexpression of A52R in a dose-dependent manner (Fig. 4 C). As Mal interacts with IRAK2 (25), we hypothesized that A52R may inhibit the activation of NF- $\kappa$ B by Mal by preventing the formation of an active Mal-IRAK2 signaling complex. Therefore, the effect of A52R expression on the ability of Mal to interact with IRAK2 was tested. As the expression of A52R increased, the amount of IRAK2 capable of coimmunoprecipitation with Mal decreased steadily (Fig. 4 D). This decrease in complex formation was not due to a decrease in the expression of either IRAK2 or Mal since direct immunoblot showed equal expression of both signaling molecules as the expression of A52R increased (unpublished data). Thus, increasing expression of A52R is able to inhibit the acti-



**Figure 5.** Gene A52R contributes to virus virulence. Groups of four female, Balb/c mice, 6-wk-old were infected intranasally with  $10^4$  PFU of vA52R-wt, v $\Delta$ A52R, or vA52R-rev. Each day animals were weighed and the signs of illness scored. Data are presented as (A) the mean weight of each group of animals compared with the mean weight of the same group on day 0, and (B) the mean signs of illness score. Error bars = SEM. Horizontal bars represent days on which there was a statistically significant difference ( $P < 0.05$ , Student's  $t$  test) between the v $\Delta$ A52R and both control groups.

vation of NF- $\kappa$ B by Mal, and this correlates with dissociation of an active Mal-IRAK2 signaling complex.

**A52R Contributes to VV Virulence.** Finally, in order to assess the importance of A52R and TLR inhibition to VV virulence, the role of A52R in the VV life cycle was investigated by the construction of a deletion mutant lacking the A52R gene and by the comparison with wild-type and revertant controls. The loss of the A52R gene did not affect the replication of the virus in cell culture or the plaque size (unpublished data). However, in a murine intranasal model the deletion mutant caused reduced weight loss (Fig. 5 A) and milder signs of illness (Fig. 5 B) compared with controls. This result was confirmed in a repeat experiment. Thus, the A52R protein contributes to virus virulence and this is likely to be due to the inhibition of TLR signaling.

Taken together, these results suggest that A52R from VV is able to inhibit TLR-induced NF- $\kappa$ B activation by associating with key signaling molecules and thus disrupting the formation of active signaling complexes, which contributes to virus virulence.

## Discussion

In this report we identify the VV protein A52R as a broad intracellular inhibitor of signaling by TLRs and show it contributes to virus virulence in vivo. In addition to inhibition of the IL-1R, the IL-18R, and TLR4 (29), A52R was shown here to inhibit NF- $\kappa$ B induction by multiple TLRs, including TLR3, which has been implicated in antiviral innate immunity.

There is intense interest in the IL-1R/TLR family, given its emerging central importance in the innate immune response to diverse pathogens (1). During the course of viral infection the body mounts several lines of host defense involving constituents of the IL-1R/TLR superfamily. The cytokines IL-1 and IL-18 are key regulators of the innate and adaptive immune response to viral infection. In particular IL-1 is responsible for inducing a fever response during viral infection, which is antagonized by the production of a soluble IL-1 $\beta$  binding protein (B15R) by VV (31). IL-18 is a potent inducer of IFN- $\gamma$ , and administration of IL-18 has been shown to elicit antiviral effects in VV-infected mice (35), while deletion of the VV IL-18 binding protein caused virus attenuation in vivo (36). As for the TLR arm of the family, recent work has suggested that they are crucial mediators of an innate immune response to viral infection. TLR3 has been shown to activate NF- $\kappa$ B and the production of IFN- $\alpha$  and IFN- $\beta$  in response to poly(I:C), a synthetic analogue of dsRNA (15), which is produced by most viruses including VV at some stage in their life cycle. In addition to its role as the receptor that responds to LPS, TLR4 has been shown to mediate the cytokine-stimulating ability of the fusion protein of respiratory syncytial virus (RSV), while RSV replicated to a higher titre and persisted longer in TLR4-deficient mice than in control mice (13). TLR4 is also required by murine retroviruses for B cell activation (14). TLR2 signaling has recently been shown to be activated by the hemagglutinin protein from measles virus (17), while activation of TLR7 by certain imidazoquinoline compounds has been shown to account for the antiviral effects of these compounds (16). It is possible that other TLRs will also have a role in responding to viral infection. If the TLR family is truly important in antiviral host defense, one would assume that viral mechanisms to antagonize this family exist. Therefore, the discovery of VV A52R as an intracellular global inhibitor of TLR signaling lends strong support to the emerging role of TLRs in the host response to viral infection.

A52R could be coimmunoprecipitated with either IRAK2 or TRAF6. The strength of the interaction with each partner suggest that each interaction is direct, rather than A52R interacting with one protein via a 'bridge' with the other. This was theoretically possible since IRAK2 and TRAF6 interact (23). Importantly, the interaction of A52R with IRAK2 was able to disrupt IRAK2/Mal interactions, while the interaction with TRAF6 disrupted TRAF6/TAK1 interactions. An important control was a lack of effect on TAB1/TAK1 complexes, showing that the effect of A52R on TRAF6/TAB1 was via TRAF6 and not TAB1.

The work here does not exclude the possibility that A52R might disrupt other important TRAF6- (and IRAK2-) containing signaling complexes, and thus increase the range of downstream signals blocked by vaccinia. Disruption of signaling complexes by A52R provides a mechanistic explanation for its ability to inhibit TIR-dependent signaling. Some viral proteins, such as the hepatitis C virus nonstructural 5A protein (37) and the rotavirus capsid protein VP4 (38) are known to affect intracellular signaling by targeting TRAF2. However, this is the first demonstration of a viral protein targeting TRAF6. The observation that the TRAF domain of TRAF6 is targeted is of interest. The TRAF domain is responsible for mediating the oligomerization of TRAF6, which is required for its ubiquitination and activation of IKK via TAK (27, 39). A dominant negative version of TRAF6 composed of only the TRAF domain is thought to function by inhibiting oligomerization (33), and it is possible that the binding of A52R functions in a similar manner.

A52R is also the first viral protein identified to target IRAK2. This, together with our demonstration of a role for IRAK2 in all the TLR pathways tested, including TLR3, suggests an important role for IRAK2 in antiviral immunity and TLRs in general. The death domain of IRAK2 was required for the association. Functionally there is a clear difference between the death domain of IRAK and IRAK2. Based on results here, IRAK2 might be predicted to be more important for TLRs involved in the antiviral response than IRAK. Determination of the exact role of IRAK2 in antiviral immunity and TLR signaling awaits the generation of an IRAK2 knockout mouse.

The apparent redundant targeting of two signaling molecules present on common pathways may indicate the critical importance to the virus of inhibiting NF- $\kappa$ B activated by TLRs. However, from recent work using knockout mouse studies it has become clear that although the IL-1R/TLR family share a common pool of downstream signaling molecules, specific molecules are used in different contexts leading to the range of different signals that are generated by TLRs. In response to LPS, TLR4 activates cytokine release from dendritic cells by a MyD88-dependent pathway, whereas NF- $\kappa$ B activation and expression of costimulatory molecules can occur in the absence of MyD88 (40). We recently identified Mal, a novel TIR containing adaptor protein, which interacts with IRAK2 and can activate NF- $\kappa$ B in response to TLR4 stimulation (25). Consistent with the targeting of IRAK2 by A52R, signaling triggered by Mal was sensitive to inhibition by A52R. Significantly, the MyD88-independent pathway, which may involve this Mal-IRAK2 axis (41), leads to the activation of the antiviral transcription factor IFN-regulatory factor 3 (IRF3) and induction of IRF3-responsive genes, and has been shown to be independent of TRAF6 (42). Thus, there would appear to be at least two types of signaling pathways activated by the IL-1R/TLR superfamily, those dependent on MyD88, IRAK/IRAK2, and TRAF6, and those activated by a Mal/IRAK2-dependent and TRAF6-independent pathway. Thus, the ability of A52R

to target both IRAK2 and TRAF6 would significantly increase the range of TIR domain-activated signaling pathways that VV is able to inhibit.

The importance of A52R, and by extrapolation TLRs, to virus virulence was demonstrated by deletion of A52R from VV, which reduced virulence. The attenuation seen by deletion of A52R is similar to that resulting from loss of IL-18 binding protein (36) or the type I IFN binding protein (43).

In conclusion, we have identified the VV protein A52R as an inhibitor of TLR signaling, which acts by a novel mechanism of targeting the two key signaling molecules IRAK2 and TRAF6. The targeting of these two molecules involved in a diverse range of TIR domain-dependent signaling pathways is likely to confer VV with a significant selective advantage in the presence of the host immune system. This study further highlights the emerging importance of TLRs in antiviral innate immunity.

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## References

1. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2:675–680.
2. Bowie, A., and L.A.J. O'Neill. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J. Leukoc. Biol.* 67:508–514.
3. Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in the Tlr4 gene. *Science*. 282:2085–2088.
4. Qureshi, S.T., L. Lariviere, G. Leveque, S. Clermont, K.J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* 189:615–625.
5. Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 410: 1099–1103.
6. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognises bacterial DNA. *Nature*. 408:740–745.
7. Brightbill, H.D., D.H. Libraty, S.R. Krutzik, R.-B. Yang, J.T. Belisle, J.R. Bleharski, M. Maitland, M.V. Norgard, S.E. Plevy, S.T. Smale, et al. 1999. Host defense mechanisms trig-

- gered by microbial lipoproteins through Toll-like receptors. *Science*. 285:732–736.
8. Aliprantis, A.O., R.-B. Yang, M.R. Mark, S. Suggett, B. Devaux, J.D. Radolf, G.R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science*. 285:736–739.
  9. Underhill, D.M., A. Ozinsky, A.M. Hajjar, A. Stevens, C.B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature*. 401:811–815.
  10. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity*. 11: 443–451.
  11. Ozinsky, A., D.M. Underhill, J.D. Fontenot, A.M. Hajjar, K.D. Smith, C.B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA*. 97:13766–13771.
  12. Takeuchi, O., T. Kawai, P.F. Muhlradt, M. Morr, J.D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13:933–940.
  13. Kurt-Jones, E.A., L. Popova, L. Kwinn, L.M. Haynes, L.P. Jones, R.A. Tripp, E.E. Walsh, M.W. Freeman, D.T. Golenbock, L.J. Anderson, and R.W. Finberg. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol.* 1:398–401.
  14. Rassa, J.C., J.L. Meyers, Y. Zhang, R. Kudravalli, and S. Ross. 2002. Murine retroviruses activate B cells via interaction with toll-like receptor 4. *Proc. Natl. Acad. Sci. USA*. 99: 2281–2286.
  15. Alexopoulou, L., A. Czopik-Holt, R. Medzhitov, and R. Flavell. 2001. Recognition of double stranded RNA and activation of NF- $\kappa$ B by Toll-like receptor 3. *Nature*. 413:732–738.
  16. Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3:196–200.
  17. Bieback, K., E. Lien, I.M. Klagge, E. Avota, J. Schneider-Schaulies, W.P. Duprex, H. Wagner, C.J. Kirschning, V. ter Meulen, and S. Schneider-Schaulies. 2002. Hemagglutinin protein of wild-type measles virus activates Toll-like receptor 2 signaling. *J. Virol.* 76:8729–8736.
  18. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. *Immunity*. 9:143–150.
  19. Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P.F. Muhlradt, and S. Akira. 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasma lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164:554–557.
  20. Schnare, M., A.C. Holt, K. Takeda, S. Akira, and R. Medzhitov. 2000. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* 10:1139–1142.
  21. Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C.A. Janeway. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell*. 2:253–258.
  22. Wesche, H., W.J. Henzel, W. Shillinglaw, S. Li, and Z. Cao. 1997. MyD88: An adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*. 7:837–847.
  23. Muzio, M., J. Ni, P. Feng, and V.M. Dixit. 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science*. 278:1612–1615.
  24. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*. 11:115–122.
  25. Fitzgerald, K.A., E.M. Palsson-McDermott, A.G. Bowie, C.A. Jefferies, A.S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M.T. Harte, et al. 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature*. 413:78–83.
  26. Hornig, T., G.M. Barton, and R. Medzhitov. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol.* 2:835–841.
  27. Wang, C., L. Deng, M. Hong, G.R. Akkaraju, J. Inoue, Z.J. Chen, and C. Wang. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*. 412:346–351.
  28. Alcami, A., and U.H. Koszinowski. 2000. Viral mechanisms of immune evasion. *Immunol. Today*. 21:447–455.
  29. Bowie, A., E. Kiss-Toth, J.A. Symons, G.L. Smith, S.K. Dower, and L.A.J. O'Neill. 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and Toll-like receptor signaling. *Proc. Natl. Acad. Sci. USA*. 97:10162–10167.
  30. Falkner, F.G., and B. Moss. 1990. Transient dominant selection of recombinant vaccinia viruses. *J. Virol.* 64:3108–3111.
  31. Alcami, A., and G.L. Smith. 1992. A soluble receptor for interleukin-1 $\beta$  encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell*. 71:153–167.
  32. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature*. 388:394–397.
  33. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D.V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature*. 383:443–446.
  34. Hsu, H., H.-B. Shu, M.-G. Pan, and D.V. Goeddel. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*. 84:299–308.
  35. Tanaka-Kataoka, M., T. Kunikata, S. Takayama, K. Iwaki, K. Ohashi, M. Ikeda, and M. Kurimoto. 1999. In vivo antiviral effect of interleukin 18 in a mouse model of vaccinia virus infection. *Cytokine*. 11:593–599.
  36. Symons, J.A., E. Adams, P.C. Reading, H. Waldmann, and G.L. Smith. 2002. The vaccinia virus C12L protein inhibits mouse IL-18 and promotes virulence in a mouse intranasal model. *J. Gen. Virol.* 83:2833–2844.
  37. Park, K.-J., S.-H. Choi, S.Y. Lee, S.B. Hwang, and M.M.C. Lai. 2002. Nonstructural 5A protein of hepatitis C virus modulates TNF- $\alpha$ -stimulated NF- $\kappa$ B activation. *J. Biol. Chem.* 277:13122–13128 First published on January 30, 2002. 10.1074/jbc.M111599200.
  38. LaMonica, R., S.S. Kocer, J. Nazarova, W. Dowling, E. Gei-

- monen, R.D. Shaw, and E.R. Mackow. 2001. VP4 differentially regulates TRAF2 signaling, disengaging JNK activation while directing NF- $\kappa$ B to effect rotavirus-specific cellular responses. *J. Biol. Chem.* 276:19889–19896.
39. Baud, V., Z.G. Liu, B. Bennett, N. Suzuki, Y. Xia, and M. Karin. 1999. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev.* 13:1297–1308.
40. Kaisho, T., O. Takeuchi, T. Kawai, K. Hoshino, and S. Akira. 2001. Endotoxin-induced maturation of Myd88-deficient dendritic cells. *J. Immunol.* 166:5688–5694.
41. Shinobu, N., T. Iwamura, M. Yoneyama, K. Yamaguchi, W. Suhara, Y. Fukuhara, F. Amano, and T. Fujita. 2002. Involvement of TIRAP/MAL in signalling for the activation of interferon regulatory factor 3 by lipopolysaccharide. *FEBS Lett.* 517:251–256.
42. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P.F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167: 5887–5894.
43. Symons, J.A., A. Alcamí, and G.L. Smith. 1995. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell.* 81:551–560.



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