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Vaccinia Virus Protein A52R Activates p38 Mitogen-activated Protein Kinase and Potentiates Lipopolysaccharide-induced Interleukin-10*

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Geraldine Maloney, Martina Schröder, and Andrew G. Bowie‡

From the Viral Immune Evasion Group, School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland

Vaccinia virus (VV) has many mechanisms to suppress and modulate the host immune response. The VV protein A52R was previously shown to act as an intracellular inhibitor of nuclear factor κB (NFκB) signaling by Toll-like receptors (TLRs). Co-immunoprecipitation studies revealed that A52R interacted with both tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 2 (IRAK2). The effect of A52R on signals other than NFκB was not determined. Here, we show that A52R does not inhibit TLR-induced p38 or c-Jun amino N-terminal kinase (JNK) mitogen activating protein (MAP) kinase activation. Rather, A52R could drive activation of these kinases. Two lines of evidence suggested that the A52R/TRAF6 interaction was critical for these effects. First, A52R-induced p38 MAP kinase activation was inhibited by overexpression of the TRAF domain of TRAF6, which sequestered A52R and inhibited its interaction with endogenous TRAF6. Second, a truncated version of A52R, which interacted with IRAK2 and not TRAF6, was unable to activate p38. Because interleukin 10 (IL-10) production is strongly p38-dependent, we examined the effect of A52R on IL-10 gene induction. A52R was found to be capable of inducing the IL-10 promoter through a TRAF6-dependent mechanism. Furthermore, A52R enhanced lipopolysaccharide/TLR4-induced IL-10 production, while inhibiting the TLR-induced NFκB-dependent genes IL-8 and RANTES. These results show that although A52R inhibits NFκB activation by multiple TLRs it can simultaneously activate MAP kinases. A52R-mediated enhancement of TLR-induced IL-10 may be important to virulence, given the role of IL-10 in immunoregulation.

The recently described Toll-like receptor (TLR)[1] family are critical in initiating an appropriate innate immune response to infectious agents, and in directing the later adaptive response. To date, 13 members of the TLR family have been identified in mammals. The TLRs belong to a superfamily that includes the interleukin 1 (IL-1) receptors. This family share significant homology in their cytoplasmic regions, which is defined by the presence of a Toll-IL-1 receptor-resistance domain (TIR) (1).

Similar to the IL-1R, engagement of the TLRs with their ligands leads to activation of several intracellular signal transduction pathways, culminating in the induction of proinflammatory cytokines such as IL-1 and tumor necrosis factor (TNF), of chemokines such as IL-8 and RANTES (2), and of the immunoregulatory cytokine IL-10 (3). Among the most prominent and best characterized of these intracellular signaling pathways are those leading to the activation of mitogen-activated protein (MAP) kinases and the transcription factor NFκB. Triggering of the IL-1R or of TLRs causes TIR adaptor molecules to be recruited to the receptor complex such as MyD88 (4) and TIR domain containing adaptor inducing interferon-β (TRIF) (5, 6). Subsequently, the IL-1 receptor-associated kinases (IRAKs) such as IRAK1, IRAK2, and IRAK4 are activated, which then engage with TRAF6, ultimately activating the IκB kinase complex. This complex phosphorylates the inhibitory molecule IκB, which leads to NFκB entering the nucleus and inducing target gene expression (7). Activation of TRAF6 also results in the activation of TAK1 and subsequent activation of MAP kinases (p42/p44, p38, and JNK).

The TLR family is now known to be important in sensing and responding to viruses. Double-stranded RNA is a molecular pattern associated with viral infection, and TLR3 has been shown to sensitize cells to activation by poly(I:C) a synthetic double-stranded RNA analogue (8). Other TLRs involved in sensing viral infection include TLR7 and TLR8, which detect single-stranded RNA from influenza, human immunodeficiency virus, and vesicular stomatitis virus (9–11), and TLR9, which recognizes genomic DNA of herpes simplex virus-2 (10, 12). Further evidence for a role for TLRs in responding to viruses comes from the fact that proteins from VV have been identified that can block TLR function (13–16). The VV genome contains numerous genes encoding proteins involved in immunomodulation and immunoevasion. For example, the virus encodes proteins that act as decoy receptors for IL-1, IL-18, and TNF (17). VV is a member of the Poxviridae, a family of complex DNA viruses that replicate in the cytoplasm of vertebrate and invertebrate cells. The most notorious member, variola virus, causes smallpox. This disease was eradicated using prophylactic inoculations with the antigenically related VV (18).

One VV protein implicated in the evasion of the host TLR response is A52R. A role for A52R in VV virulence has been clearly established in that deletion of *a52r* from VV led to an attenuated virus in a murine intranasal infection (15). A52R was shown to be capable of interacting with IRAK2 and TRAF6

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Trinity College Dublin, Dublin 2, Ireland. Tel.: 353-1-6082435; Fax: 353-1-6772400; E-mail: agbowie@tcd.ie.

[1] The abbreviations used are: TLR, Toll-like receptor; IL-1, interleukin 1; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun amino N-terminal kinase; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; MEF, murine embryonic fibroblasts; TIR, Toll-interleukin-1 receptor-resistance domain; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor-associated factor 6; VV, vaccinia virus; TAK1, transforming growth factor-β activated kinase 1; NFκB, nuclear factor κB.

and to block every IL-1R-TLR pathway to NFκB activation tested (13, 15). However, the effect of A52R on signals other than NFκB has not been determined. Given that many viral proteins have multiple activities and that other intracellular effects of A52R might contribute to its role in virulence, we tested the effect of A52R on signals other than NFκB. Here we show that A52R can activate the MAP kinases p38 and JNK in a TRAF6-dependent manner. In addition, A52R leads to enhancement of the TLR-induced p38-dependent gene IL-10. In contrast, inhibition of the TLR-induced NFκB-dependent genes IL-8 and RANTES is observed. These results highlight the ability of A52R to differentially modulate TLR signaling. This ability of A52R to activate p38 and potentiate TLR-induced IL-10 may be important to its role in virulence.

MATERIALS AND METHODS

Expression and Reporter Plasmids—CD4-TLR4 was a kind gift from R. Medzhitov (Yale University, New Haven, CT), and TLR3 was kindly provided by D. Golenbock (University of Massachusetts Medical School, Worcester, MA). Myc-IRAK2 was a gift from M. Muzio (19). The TRAF6 expression plasmids, FLAG-TRAF6 and FLAG-TRAF domain (ΔTRAF6, amino acids 289–522), were provided by Tularik Inc. (San Francisco, CA). A52R expression plasmid was previously described (13). ΔA52R was generated by PCR of the A52R plasmid and comprised amino acids 1–144 of the wild type protein (which is 190 amino acids in length), plus an extra 27 amino acids derived from the vector sequence. The NFκB luciferase reporter construct was a gift from R. Hofmeister (University of Regensburg, Germany). The human IL-10 promoter reporter plasmid was a kind gift from L. Ziegler-Heitbrock (20).

Antibodies and Reagents—Anti-FLAG M2 monoclonal antibody and anti-Myc monoclonal antibody clone 9E10 were purchased from Sigma. Anti-A52R antibody was previously described (15). Anti-IκBα antibody was provided by R. Hay (University of St. Andrews, Scotland). Other antibodies used were anti-TRAF6 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) antibody and anti-p38 MAP kinase antibody (both from Cell Signaling Technology, Beverly, MA), and anti-rabbit IgG antibody-$F(ab)_2$ fragment (Abcam, Cambridge, United Kingdom).

Human rIL-1α was from the National Cancer Institute (Frederick, MD). The synthetic double-stranded RNA analogue, poly(I:C), was purchased from Amersham Biosciences (Bucks, UK). Lipopolysaccharide from *Escherichia coli* serotype EH100(Ra) and the p38 MAP kinase inhibitor SB203580 were purchased from Alexis Biochemicals (Bingham, Nottingham, UK).

Reporter Gene Assays—HEK 293 cells (2×10^4 cells per well) were seeded into 96-well plates and transfected 24 h later with expression vectors and the indicated luciferase reporter genes using GeneJuice™ (Novagen) according to the manufacturer's instructions. In all cases, 20 ng/well of phRL-TK reporter gene (Promega) was co-transfected to normalize the data for transfection efficiency. The total amount of DNA per transfection was kept constant at 230 ng by addition of pcDNA3.1 (Stratagene). For NFκB assays, 60 ng of a NFκB luciferase reporter gene was used (15). For IL-10 promoter assays, 60 ng of the IL-10 promoter luciferase reporter gene was used. For MAP kinase reporter assays the PathDetect System™ (Stratagene) was used, whereby either 0.25 ng of a c-Jun-Gal4 (to assay JNK) or a CHOP-Gal4 (to assay p38 MAP kinase) fusion vector was used in combination with 60 ng of pFR-luciferase reporter. Cells were stimulated with 100 ng/ml IL-1, 1 μg/ml LPS, or 25 μg/ml poly(I:C), where indicated 6 h prior to harvesting. After 24 h, reporter gene activity was measured (15). Data are expressed as mean -fold induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each preformed in triplicate.

Immunoprecipitation and Immunoblotting—HEK 293T cells (1.5×10^6) were seeded into 10-cm dishes 24 h prior to transfection. Transfections were carried out using GeneJuice™. For coimmunoprecipitations, 4 μg of each construct were transfected. Where only one construct was transfected the total amount of DNA (8 μg) was kept constant by supplementation with pcDNA3.1. Cells were harvested 24 h post-transfection, washed twice in phosphate-buffered saline, and lysed in 850 μl of lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40 containing 1 mM phenylmethylsulfonyl fluoride, 0.01% (v/v) aprotinin, and 1 mM sodium orthovanadate). For immunoprecipitation, the indicated antibodies were precoupled to either protein A-Sepharose (polyclonal and FLAG monoclonal antibodies) or protein G-Sepharose beads (all other monoclonal antibodies) overnight at 4 °C. The beads were then washed twice in lysis buffer and incubated with the cell lysates overnight at 4 °C. The immune complexes were washed, boiled with 30 μl of 3× sample buffer (62.5 mM Tris, 2% (w/v) SDS, 10% v/v glycerol, 0.1% (w/v) bromphenol blue), and analyzed using standard SDS-PAGE and Western blotting techniques.

For analysis of p38 MAP kinase activation by Western blot, a specific antibody raised against phosphorylated p38 (Thr^{180}/Tyr^{182}) was employed. Total levels of p38 MAP kinase protein were also analyzed using anti-p38 MAP kinase antibody. HEK 293 cells (1×10^5 cells per well) were seeded into 6-well plates and transfected 24 h later with ΔTRAF6 or A52R encoding plasmids as indicated, using GeneJuice™. The total amount of DNA (2.3 μg) was kept constant by supplementation with pcDNA3.1. 24 h after transfection, cells were lysed in 100 μl of SDS sample buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 50 mM dithiothreitol, 10% glycerol, 0.1% bromphenol blue). Lysates were then resolved by SDS-PAGE, transferred to poly(vinylidene difluoride) membranes, and probed with the indicated antibodies according to the manufacturer's instructions.

Determination of Cytokine Concentrations—HEK 293 clonal cell lines stably expressing either TLR4/MD-2 (HEK-TLR4) or TLR3 (HEK-TLR3) (21) or the murine macrophage cell line RAW 264.7 were used for determination of cytokine production. Cells (2×10^4 cell per well) were seeded into 96-well plates and transfected 24 h later with an expression plasmid encoding A52R, TRAF6, or ΔTRAF6 using GeneJuice™ where indicated. 24 h after transfection, cells were stimulated with 1 μg/ml LPS or 25 μg/ml poly(I:C). 2 h prior to stimulation the p38 MAP kinase inhibitor SB203580 was added were indicated. After 24 h supernatants were harvested, and IL-8, RANTES, or IL-10 concentrations were determined by enzyme-linked immunosorbent assay (R&D Biosystems). Experiments were performed three times in triplicate, and data are expressed as mean ± S.D. from one representative experiment.

RESULTS

A52R Drives p38 MAP Kinase Activation—We previously showed that deletion of the vaccinia virus *a52r* gene reduced virus virulence in a murine intranasal infection model, that A52R acts as a potent inhibitor of NFκB activation induced by IL-1 and various TLRs, and that it was capable of interacting with both TRAF6 and IRAK2 (15). However, the effect of A52R on signals other than NFκB was not determined and thus there might be other functions of A52R that also contribute to virulence. Therefore, here we examined the effect of A52R on p38 MAP kinase activation. For this we used the Stratagene PathDetect™ System that is based on the ability of p38 MAP kinase to phosphorylate and activate the transcription factor CHOP. This is assayed by an increase in the ability of the Gal4-CHOP fusion protein to transactivate the pFR luciferase reporter, which contains Gal4 binding sites in its promoter. Fig. 1*A* shows that treatment of cells with IL-1, ectopic expression of CD4-TLR4, or ectopic expression of TLR3 together with poly(I:C) stimulation led to activation of p38 MAP kinase. Surprisingly, ectopic expression of increasing amounts of a plasmid encoding A52R enhanced IL-1-, CD4-TLR4-, and TLR3-mediated p38 MAP kinase activation (Fig. 1*A*). This was in contrast to the inhibitory effect of A52R on IL-1/TLR-induced NFκB activation (Fig. 1*B* and Refs. 13 and 15). In fact, expression of A52R alone in unstimulated cells led to both p38 MAP kinase and JNK activation (Fig. 1*C*), whereas A52R alone had no effect on basal levels of NFκB activity (not shown). Thus, A52R has opposite effects on IL-1/TLR-induced NFκB and MAP kinase activation and can in fact activate MAP kinases in the absence of any other stimulus.

Activation of p38 by A52R Requires Interaction with TRAF6—Activation of p38 by A52R could conceivably be because of its ability to interact with either IRAK2 or TRAF6 (15). To determine whether this was the case, we began to generate truncated versions of A52R to map the sites of interaction between A52R and TRAF6 and IRAK2. A truncated version of A52R lacking 46 amino acids at the C-terminal was constructed (Fig. 2*A*). This truncated A52R protein, here termed ΔA52R, was

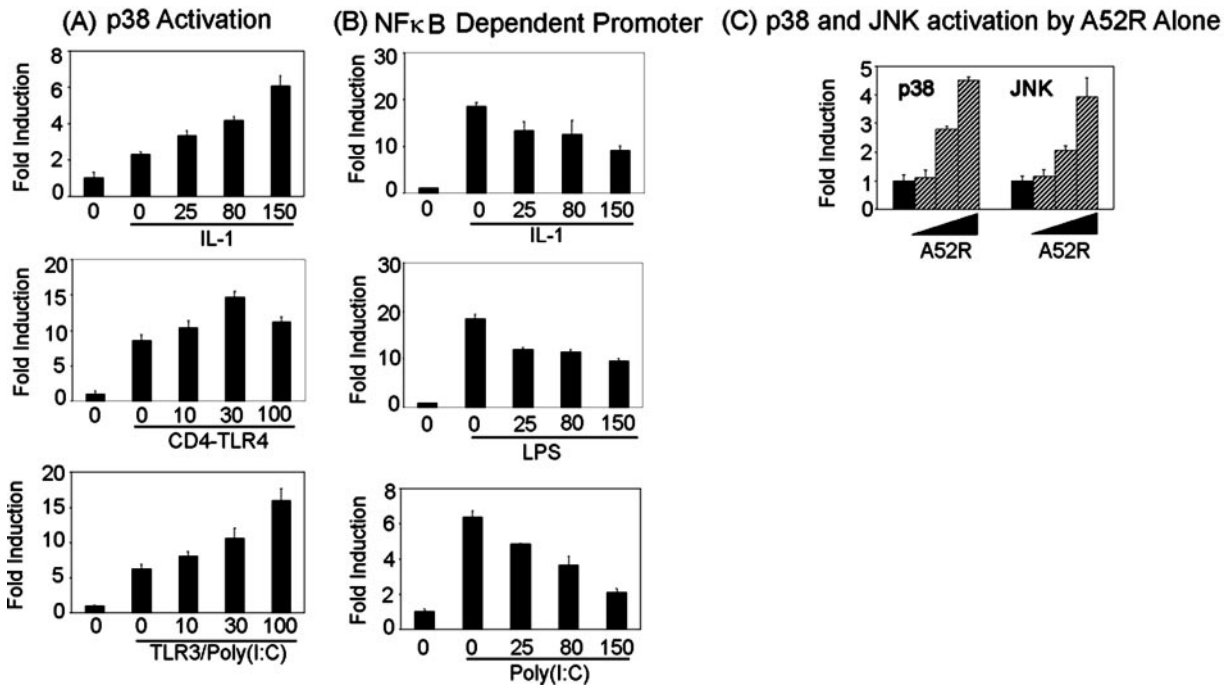

FIG. 1. **A52R drives p38 MAP kinase activation.** *A*, HEK 293 cells were transfected with increasing amounts (ng) of a plasmid encoding A52R as indicated, the phRL-TK reporter gene, 0.25 ng of CHOP Gal4 fusion vector and pFR luciferase reporter plasmids as described under "Materials and Methods." *B*, HEK 293 cells stably expressing pcDNA 3.1, TLR3, or TLR4 were transfected with increasing amounts (ng) of A52R as indicated, the phRL-TK reporter gene and the NFκB reporter plasmid. Six hours prior to harvesting, the cells were stimulated with 100 ng/ml IL-1, 25 μg/ml poly(I:C), or 1 μg/ml LPS as indicated, and luciferase gene activity was measured. *C*, HEK 293 cells were transfected with increasing amounts (10, 30, and 100 ng) of a plasmid encoding A52R, the phRL-TK reporter gene, 0.25 ng of CHOP Gal4 fusion vector, or 0.25 ng of c-Jun Gal4 fusion vector and pFR luciferase and harvested 24 h later. Data are expressed as mean -fold induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

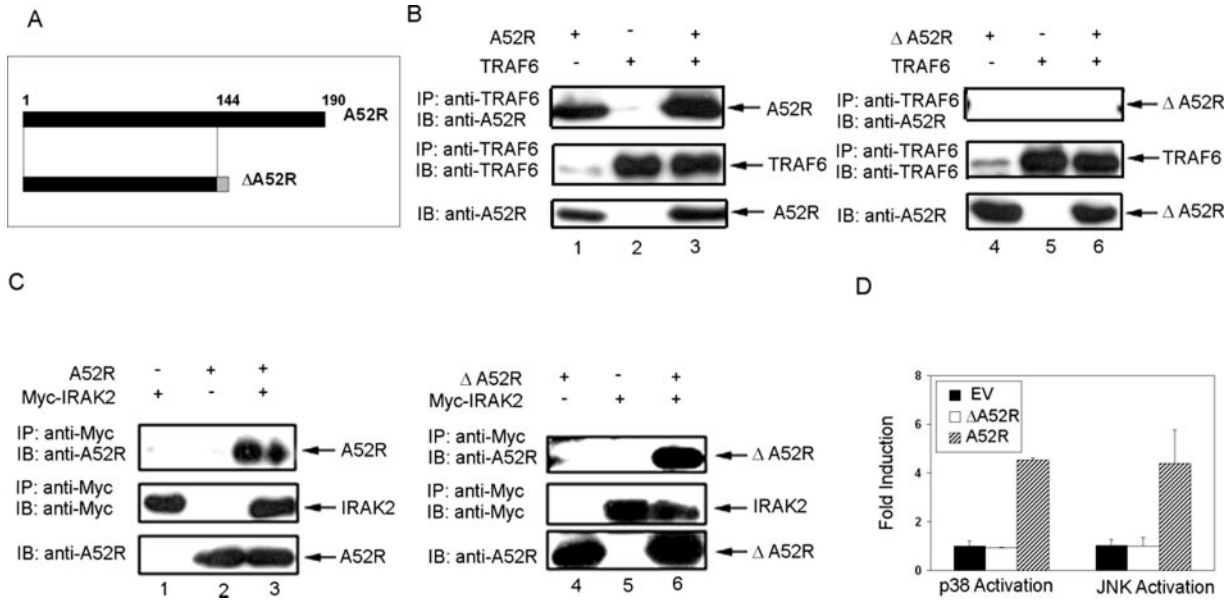

FIG. 2. **A52R activates MAP kinases via the TRAF6 interaction.** *A*, schematic representation of A52R and ΔA52R. *B* and *C*, HEK 293T cells were transfected with 4 μg of TRAF6 (*B*) or 4 μg of Myc-IRAK2 (*C*) and 4 μg of A52R (*left panel*) or ΔA52R (*right panel*) as indicated. After 24 h, lysates were subjected to immunoprecipitation (*IP*) and subsequent immunoblotting (*IB*) with the indicated antibodies. Data are representative of one experiment from a minimum of three separate experiments. *D*, HEK 293 cells were transfected with 100 ng of plasmid encoding pcDNA3.1 (*EV*) or A52R or ΔA52R, phPL-TK reporter gene, 0.25 ng of CHOP Gal4 fusion vector, or 0.25 ng of c-Jun Gal4 fusion vector and pFR luciferase reporter plasmids, as described under "Materials and Methods." After 24 h the cells were harvested and the reporter gene activity measured. Data are expressed as mean -fold induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

detectable by the anti-A52R antibody and was expressed at similar levels to A52R (Fig. 2, *B* and *C*). To determine whether ΔA52R was still capable of interacting with TRAF6 and/or IRAK2, co-immunoprecipitations were carried out. ΔA52R was unable to form a complex with TRAF6 but retained its ability to interact with IRAK2. A co-immunoprecipitation with anti-TRAF6 antibody pulled down A52R with both endogenous TRAF6 (Fig. 2*B*, *top panel*, *lane 1*) and overexpressed TRAF6

(Fig. 2*B*, *top panel*, *lane 3*). In contrast, under the same conditions ΔA52R was not detected in complex with either endogenous or overexpressed TRAF6 (Fig. 2*B*, *top panel*, *lanes 4* and *6*). However, a clear interaction between ΔA52R and IRAK2 was observed (Fig. 2*C*). The ability of ΔA52R and A52R to form a complex with IRAK2 appeared equal (Fig. 2*C*, *top panels*, compare *lanes 3* and *6*). Next, the ability of ΔA52R to induce MAP kinase activation was tested. Fig. 2*D* shows that in contrast to A52R, ΔA52R failed to drive p38 and JNK activation. Thus, the failure of ΔA52R to activate p38 and JNK correlated with its inability to interact with TRAF6, suggesting a role for TRAF6 in the stimulatory effect of A52R on MAP kinases.

A52R Activates p38 via the TRAF Domain of TRAF6—We next sought to examine more closely how the interaction between A52R and TRAF6 was responsible for the p38 MAP kinase activation. Fig. 3*A* shows that consistent with previous work (15), A52R interacted with the TRAF domain of TRAF6 (amino acids 289–522). The TRAF domain of TRAF6, here termed ΔTRAF6, has been reported to act as a dominant negative, inhibiting IL-1-induced p38 and JNK activation (22). We therefore hypothesized that if the A52R-TRAF domain interaction was important for A52R-induced p38 activation, overexpression of ΔTRAF6 would sequester A52R and prevent p38 MAP kinase activation. To test this we examined p38 activation by Western blot analysis in HEK 293 cells using a phospho-specific p38 antibody. Fig. 3*B* shows that transfection of cells with ΔTRAF6 alone had no effect on levels of phospho-p38 (*upper panel*, *lane 2*). Consistent with the reporter gene based assay (Fig. 1), overexpression of A52R led to an increase in phospho-p38 (Fig. 3*B*, *upper panel*, compare *lane 3* to *lane 1*), whereas ΔA52R expression had no effect on phospho-p38 (not shown). Co-expression of ΔTRAF6 with A52R completely inhibited A52R-mediated p38 activation (Fig. 3*B*, *upper panel*, *lane 4*). As a control, A52R was shown to have no effect on IκBα protein levels (Fig. 3*B*, *third panel*), and similarly did not lead to an increase in IκBα phosphorylation (not shown). Inhibition of p38 MAP kinase activation by overexpression of ΔTRAF6 was likely because of ΔTRAF6 sequestering A52R and preventing its interaction with endogenous TRAF6. Fig. 3*C* shows this to be the case because the presence of overexpressed ΔTRAF6 inhibited the interaction of A52R with endogenous TRAF6 (compare *lanes 4* and *2*). Thus A52R activates p38 by engaging the TRAF domain of TRAF6.

A52R Enhances TLR-induced IL-10 Induction While Inhibiting NFκB-dependent Genes—Previous studies have shown that LPS-induced IL-10 production is p38 dependent (23, 24). Therefore, we wondered whether A52R would have an effect on IL-10 induction. We first examined the effect of A52R on the IL-10 promoter using a reporter gene assay. Interestingly, A52R was capable of driving the IL-10 promoter in a dose-dependent manner (Fig. 4*A*). In contrast, A52R expression did not affect the basal activity of a range of NFκB-dependent promoters, including IL-8, RANTES, and interferon-β (not shown). To examine whether the induction of the IL-10 promoter was TRAF6-dependent, we next assessed the ability of ΔTRAF6 to inhibit A52R-mediated IL-10 induction. Fig. 4*B* shows that a single dose of ΔTRAF6 was capable of negating the stimulatory effect of A52R on the IL-10 promoter. In addition, A52R failed to activate the IL-10 reporter in TRAF6$^{-/-}$ murine embryonic fibroblasts (MEFs). In the absence of TRAF6, the ability of A52R to drive the IL-10 promoter was abolished compared with a 3-fold induction in normal MEFs (Fig. 4*C*), thus implicating TRAF6 in the activation of the IL-10 promoter by A52R. We next used the murine macrophage cell line RAW 264.7 to analyze the effect of A52R-induced IL-10 protein production. The p38 inhibitor SB203580 has previously been shown to inhibit

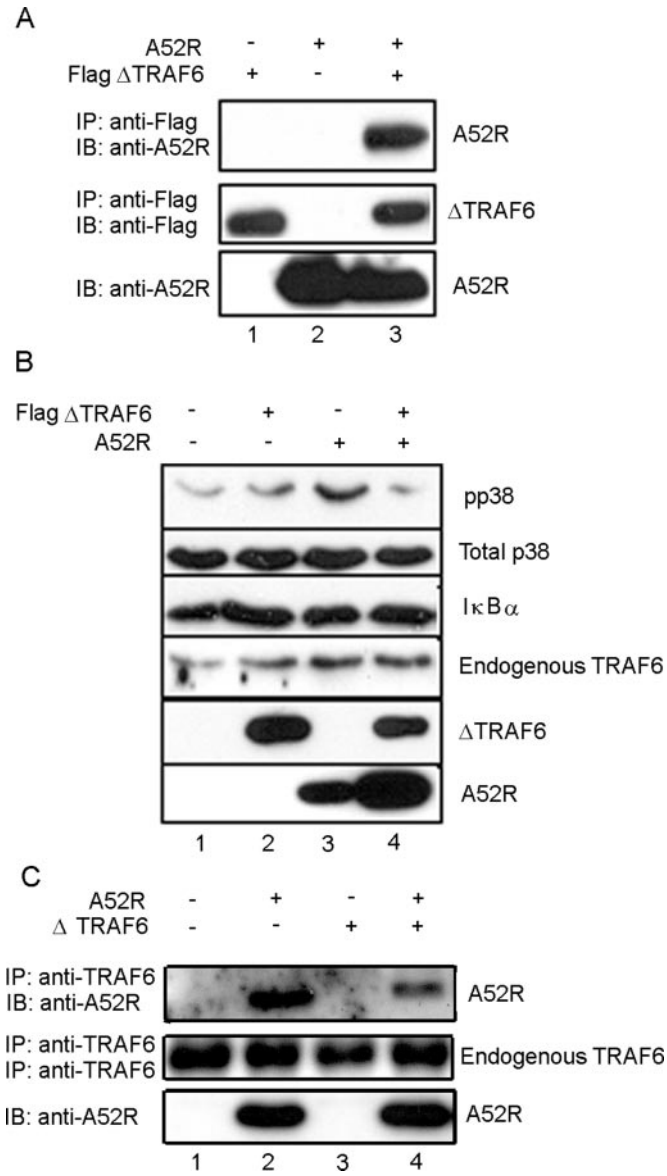

FIG. 3. **A52R activates p38 via the TRAF domain of TRAF6.** *A*, HEK 293T cells were transfected with 4 μg of A52R and 4 μg of FLAG-ΔTRAF6 as indicated. After 24 h lysates were subjected to immunoprecipitation (*IP*) and subsequent SDS-PAGE and immunoblotting (*IB*) with the indicated antibodies. Whole cell lysates were analyzed for expression of A52R. *B*, HEK 293 cells were seeded into a 6-well plate. 24 h later the cells were transfected with 1150 ng of expression plasmids ΔTRAF6 and/or A52R as indicated. Cells were harvested 24 h later, and lysates were subjected to SDS-PAGE and immunoblotting with antibodies to the indicated protein. *C*, HEK 293 cells were transfected with 0.5 μg of A52R and 7.5 μg of ΔTRAF6 as indicated. After 24 h, lysates were subjected to immunoprecipitation and subsequent immunoblotting with the indicated antibodies. Whole cell lysates were analyzed for expression of A52R. Data are representative of one experiment from two separate experiments.

LPS/TLR4-induced IL-10 production in human peripheral blood mononuclear cells (23) and in the human monocyte cell line THP-1 (24). Consistent with this, here SB203580 inhibited LPS-induced IL-10 production (Fig. 5*A*, *right panel*). Expression of A52R alone did not lead to IL-10 production in unstimulated cells (Fig. 5*A*, *left panel*). However, expression of A52R strongly enhanced LPS-induced IL-10 production (Fig. 5*A*, *left panel*). This is suggestive of a potent effect on TLR-induced IL-10 production given that only a small fraction (3% on average, not shown) of the RAW 264.7 cells stimulated by LPS to release IL-10 would be expected to be expressing A52R in this

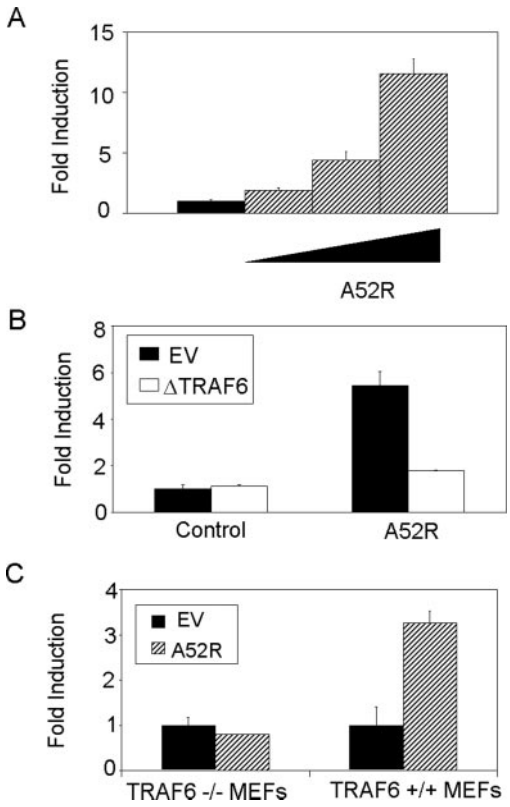

FIG. 4. **A52R drives the IL-10 promoter in a TRAF6-dependent manner.** *A* and *B*, HEK 293 cells were seeded into a 96-well plate and transfected 24 h later with the IL-10 promoter luciferase reporter plasmid, phRL-TK, and increasing amounts of A52R (10, 30, and 100 ng) (A) or 30 ng of A52R in the presence of 30 ng of a plasmid encoding ΔTRAF6 as indicated (*B*). *C*, TRAF6$^{-/-}$ MEFs or TRAF6$^{+/+}$ MEFs were seeded into a 96-well plate and transfected 24 h later with the IL-10 promoter luciferase reporter plasmid, phRL-TK, and 100 ng of A52R. After 24 h the cells were harvested and the reporter gene activity measured. Data are expressed as mean fold -induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

transient transfection system. The fact that A52R failed to drive the IL-10 promoter in TRAF6$^{-/-}$ MEFs suggested that the TRAF6-A52R interaction was critical for the effect of A52R on IL-10 production. This conclusion prompted us to examine the role of TRAF6 on LPS-induced IL-10 production. Consistent with the effect of A52R on LPS-induced IL-10, ectopic expression of TRAF6 enhanced (Fig. 5*B*, *left panel*), whereas ΔTRAF6 inhibited (Fig. 5*B*, *right panel*), LPS-induced IL-10 production. Next we sought to compare the effect of A52R on IL-10 induction to its effects on two NFκB-dependent genes, the chemokines IL-8 (25) and RANTES (26). Stimulation of HEK-TLR4 cells with LPS led to IL-8 production, whereas stimulation of HEK-TLR3 cells with poly(I:C) led to RANTES production (Fig. 5*C*). In both cases transient transfection with A52R inhibited chemokine production in a dose-dependent manner (Fig. 5*C*). Thus A52R differentially modulates TLR-induced gene expression, by potentiating the p38-dependent gene IL-10, while inhibiting the NFκB-dependent genes IL-8 and RANTES.

DISCUSSION

In this report we investigated the effect of the VV protein A52R on MAP kinase activation and LPS-induced IL-10 production. We showed that A52R expression could activate both p38 and JNK MAP kinases. Furthermore, we showed that A52R dramatically enhanced TLR4-induced IL-10 production, which is known to be p38-dependent. In contrast, inhibition of the TLR-induced NFκB-dependent chemokines IL-8 and RANTES by A52R was observed. The fact that A52R differentially modulates TLR signaling reveals a further layer of complexity in the viral subversion of TLRs.

The ability of A52R to activate p38 was dependent on TRAF6. The fact that ΔA52R, which could not form a complex with TRAF6 (Fig. 2*B*), failed to activate p38 and JNK (Fig. 2*D*) suggests that TRAF6 is crucial for mediating p38 and JNK activation by A52R (Fig. 1*C*). Furthermore, overexpression of the TRAF domain of TRAF6, which A52R interacts with, blocked the ability of A52R to activate p38, by inhibiting the interaction of A52R with endogenous TRAF6 (Fig. 3*C*). The TRAF domain of TRAF6 is responsible for mediating oligomerization of TRAF6 (22). This facilitates the interaction of TRAF6 with TRAF6-regulated IκB kinase activator 1, a dimeric ubiquitin-conjugating enzyme complex composed of Ubc13 and Uve1A (27). This interaction causes a novel form of polyubiquitination involving Lys^{63} of ubiquitin to occur. TRAF6 is ubiquitinated and TAK1 is activated, which in turn phosphorylates MAP kinase kinase 6 in the JNK-p38 kinase pathways (27). In the absence of any upstream activation, enforced oligomerization of TRAF6 leads to MAP kinase activation (22). It remains to be determined whether A52R can modulate TRAF6 ubiquitination, oligomerization, or TAK1 activation. Previously it was thought that A52R inhibited NFκB activation by disrupting the formation of a TRAF6-TAK1-TAK1-binding protein 1 complex (15). However, NFκB inhibition by A52R is more likely to be because of its ability to interact with IRAK2 (Fig. 2*C* and Ref. 15), because ΔA52R, which only interacted with IRAK2 and not TRAF6 (Fig. 2, *B* and *C*), was still capable of inhibiting TLR-induced NFκB activation (data not shown).

McCoy *et al.* (28) recently showed that a peptide derived from 11 amino acids of A52R was capable of inhibiting cytokine secretion in response to TLR activation. This study is consistent with our findings, because in Fig. 5*C* we show that full-length A52R can also inhibit TLR-induced cytokine and chemokine production. McCoy *et al.* (28) speculate, and show some data to suggest, that the inhibitory effects they observe are because of an inhibition of NFκB. Importantly, the peptide with this inhibitory capacity maps to a region in ΔA52R, suggesting that the peptide is likely to be mediating its effects via IRAK2, leading to NFκB inhibition.

The effect of A52R on the activation of the MAP kinases p38 and JNK is consistent with the emerging theme that viral proteins engage host TRAF molecules to modulate signaling pathways. For example, the non-structural 5A (NS5A) protein of hepatitis C virus interacts with the TRAF domain of TRAF2 and can potentiate TNFα-induced JNK activation (29). Another viral protein, VP4 from rotaviruses, also interacts with TRAF2. However, this protein has opposite effects to A52R in that it activates NFκB and inhibits JNK activation (30). In addition, similar to A52R, the oncogenic latent membrane protein 1 from Epstein-Barr virus has been shown to induce p38 MAP kinase through a TRAF6-dependent mechanism (31). Similar to our findings (Fig. 3*B*) overexpression of a dominant negative form of TRAF6 entirely blocked p38 MAP kinase induction by latent membrane protein 1 (31).

The ability of A52R to activate p38, together with its known inhibitory effect on NFκB activation were shown to translate into effects on gene induction by TLRs. LPS-induced IL-10 (which is a known to be p38-dependent) was enhanced in the presence of A52R (Fig. 5*A*), whereas TLR-induced NFκB-dependent chemokine production was inhibited (Fig. 5*C*). The effect of A52R on LPS-induced IL-10 was likely to be at the level of the promoter, because A52R expression led to activation of an IL-10 promoter (Fig. 4*A*). However, A52R expression did not lead to IL-10 protein in the absence of LPS (Fig. 5*A*, *left*

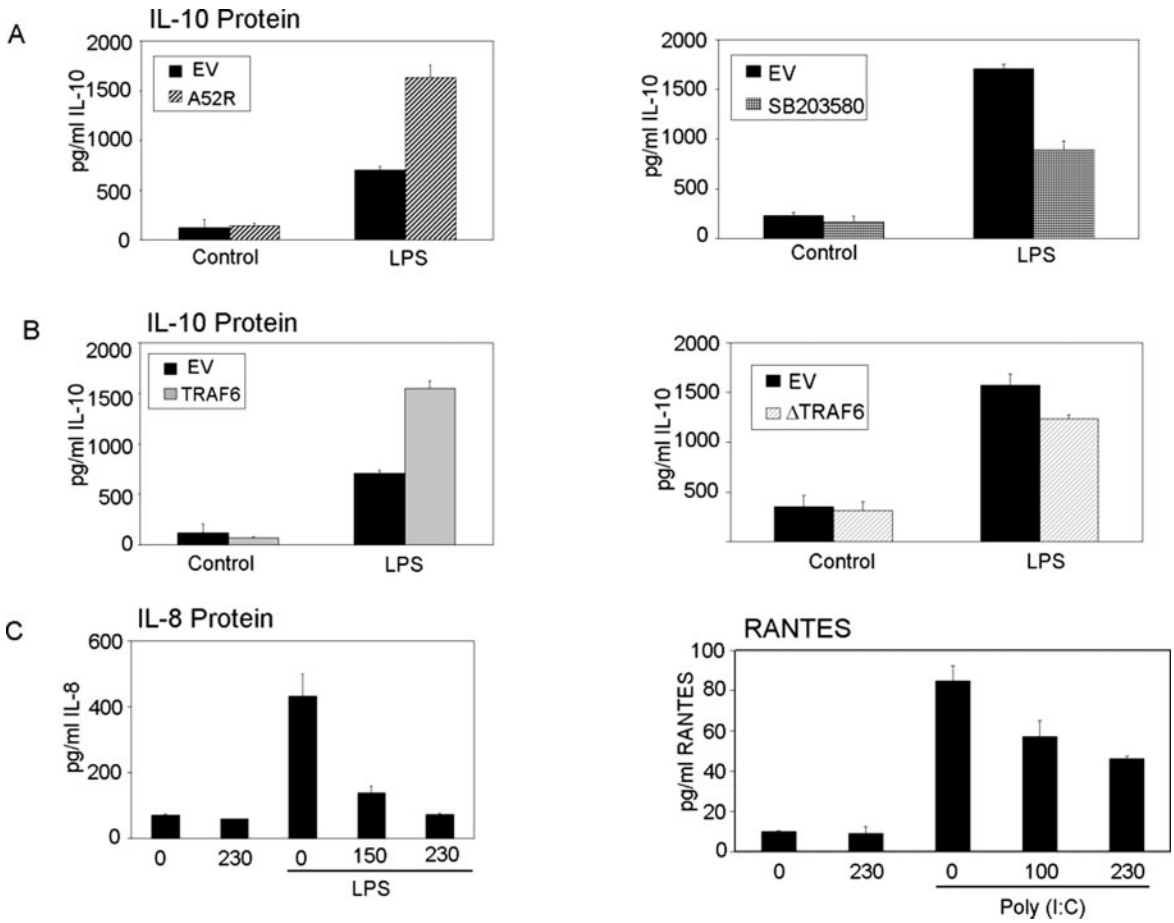

FIG. 5. **Differential effects of A52R on TLR-dependent gene induction.** *A*, murine macrophage RAW 264.7 cells were transfected with 180 ng of plasmid encoding A52R, 24 h prior to stimulation with 1 μg/ml LPS (*left panel*). Two hours after stimulation with LPS, 10 μM SB203580 was added (*right panel*). Twenty-four hours after stimulation supernatants were harvested and assayed for IL-10 by enzyme-linked immunosorbent assay. Data are expressed as picograms/ml ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate. *B*, RAW 264.7 cells were transfected with 180 ng of plasmid encoding TRAF6 (*left panel*) or ΔTRAF 6 (*right panel*) 24 h prior to stimulation with 1 μg/ml LPS. Twenty-four hours after stimulation, supernatants were harvested and assayed for IL-10 by enzyme-linked immunosorbent assay. *C*, HEK-TLR4 (*left panel*) or HEK-TLR3 (*right panel*) cells were transfected with the indicated amounts of a plasmid encoding A52R 24 h prior to stimulation with 1 μg/ml LPS (*left panel*) or 25 μg/ml poly(I:C) (*right panel*). Twenty-four hours after stimulation, supernatants were harvested and assayed for IL-8 and RANTES by enzyme-linked immunosorbent assay. Data are expressed as picograms/ml ± S.D. relative to control levels, for a representative experiment from two experiments, each performed in triplicate.

panel), suggesting that induction of IL-10 promoter by A52R was not sufficient to lead to protein expression. Rather, A52R may synergize with LPS at the level of the IL-10 promoter (presumably in a p38-dependent fashion), leading to enhanced IL-10 production when the TLR4 pathway is fully activated. TRAF6 was clearly shown to have a role in the synergistic effect, because ΔTRAF6, which inhibited the interaction of A52R with endogenous TRAF6 (Fig. 3*C*), blocked IL-10 promoter induction by A52R (Fig. 4*B*), whereas A52R could not induce the IL-10 promoter in TRAF6$^{-/-}$ MEFs (Fig. 4*C*). Furthermore, ectopic expression of TRAF6 led to a similar effect on IL-10 protein in the presence of LPS (Fig. 5*B*, *left panel*) as seen for A52R. Interestingly, IRAK1 has also recently been shown to be critically involved in LPS-induced IL-10 production (32). Furthermore, latent membrane protein 1 from Epstein-Barr virus, which as mentioned can interact with TRAF6 and potentiate p38, can induce IL-10 production (31, 33). The enhancement of IL-10 production by A52R (and indeed TRAF6) may also have a post-translational component because IL-10 mRNA contains destabilizing AU repeats in its 3′-untranslated region (34). It is known that p38 activation leads to stabilization of cytokine mRNA containing these repeats (35).

IL-10 is a pleiotropic cytokine that inhibits inflammatory and cell-mediated immune responses (3). TLR-induced IL-10 has been shown to have a role in the generation of T regulatory cells and to lead to the inhibition of Th-1 responses (36–38). TLR-mediated anti-inflammatory signals are beneficial after the elimination of pathogens; however, they can induce dangerous immunosuppressive mechanisms if activated too early during a severe infection. Recently it has become clear that TLR-induced IL-10 represents an important target of immune subversion for some pathogens. For example, the *Yersinia* virulence factor LcrV interacts with TLR2 leading to immunosuppression by induction of IL-10 (39). TLR2$^{-/-}$ mice (39) and IL-10$^{-/-}$ mice (40) are less susceptible to oral *Yersinia enterocolitica* infection than wild type controls. The fungus *Candida albicans* has been shown to trigger immunosuppression through TLR2-induced IL-10 and subsequent survival of T regulatory cells (37). In addition, immune escape of mouse mammary tumor virus, which leads to persistent infection, has been shown to be dependent on TLR4-triggered production of IL-10 (36). Therefore the enhancement of TLR4-induced IL-10 production by A52R may represent a vaccinia immune subversion mechanism. Unlike other viruses such as Epstein-Barr virus (41) and poxvirus Orf (42), VV does not encode an IL-10 homologue. However, VV replication has been shown to be impaired in IL-10$^{-/-}$ mice (43). Another study showed that VV infection induced IL-10 in human monocytes, with a more

significant increase in IL-10 expression observed following LPS treatment (44). Therefore, the ability of A52R to enhance TLR-induced IL-10 production may be contributing to the IL-10 production observed in infected human monocytes.

To date TLR2, -3, -4, -7, -8, and -9 have been shown to be activated by viral proteins and nucleic acids (8–12, 45–48). However, no TLR has yet been shown to recognize VV. Nevertheless, VV encodes other proteins that have been shown to target the IL-1R-TLR superfamily; these are A46R and N1L (13, 14). A46R is a viral TIR domain-containing protein that interacts with TIR adaptor proteins leading to inhibition of IL-1R/TLR signaling (13, 16), whereas N1L has been shown to suppress NFκB activation by IL-1R-TLRs by targeting the IKK complex (14). However, the ability to enhance TLR-induced IL-10 is unique to A52R, because overexpression of neither A46R nor N1L resulted in an increase (or decrease) in TLR-stimulated IL-10 production (not shown). Furthermore, A52R, A46R, and N1L are not functionally redundant because a single deletion of each gene affected virus virulence (15, 16, 49).

This study sheds further light on how VV interacts with the TLR system, and reveals that A52R is a multifunctional VV protein that not only inhibits TLR-mediated NFκB activation (13, 15) and NFκB-dependent genes, but can also, via TRAF6, activate p38 MAP kinase and subsequently potentiate TLR-induced IL-10. Both of these activities of A52R are likely to contribute to its role in virulence (15).

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Vaccinia Virus Protein A52R Activates p38 Mitogen-activated Protein Kinase and Potentiates Lipopolysaccharide-induced Interleukin-10

Geraldine Maloney, Martina Schröder and Andrew G. Bowie

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