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# Cytosolic DNA Triggers Mitochondrial Apoptosis via DNA Damage Signaling Proteins Independently of AIM2 and RNA Polymerase III

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A key host response to limit microbial spread is the induction of cell death when foreign nucleic acids are sensed within infected cells. In mouse macrophages, transfected DNA or infection with modified vaccinia virus Ankara (MVA) can trigger cell death via the absent in melanoma 2 (AIM2) inflammasome. In this article, we show that nonmyeloid human cell types lacking a functional AIM2 inflammasome still die in response to cytosolic delivery of different DNAs or infection with MVA. This cell death induced by foreign DNA is independent of caspase-8 and carries features of mitochondrial apoptosis: dependence on BAX, APAF-1, and caspase-9. Although it does not require the IFN pathway known to be triggered by infection with MVA or transfected DNA via polymerase III and retinoid acid-induced gene I-like helicases, it shows a strong dependence on components of the DNA damage signaling pathway: cytosolic delivery of DNA or infection with MVA leads to phosphorylation of p53 (serines 15 and 46) and autophosphorylation of ataxia telangiectasia mutated (ATM); depleting p53 or ATM with small interfering RNA or inhibiting the ATM/ATM-related kinase family by caffeine strongly reduces apoptosis. Taken together, our findings suggest that a pathway activating DNA damage signaling plays an important independent role in detecting intracellular foreign DNA, thereby complementing the induction of IFN and activation of the AIM2 inflammasome. *The Journal of Immunology*, 2012, 188: 394–403.

**T**he innate immune system recognizes invading pathogens via conserved **pathogen-associated molecular patterns (PAMPs)**. Upon recognition of a PAMP, a range of cellular

defense mechanisms are triggered including two pivotal responses: 1) **secretion of cytokines that activate the immune system and 2) induction of cell death in infected cells**. In the case of viruses and some intracellular bacteria, their **nucleic acids act as the principal PAMPs recognized**. The retinoid acid-induced gene I (RIG-I)-like helicases (RLHs) recognize cytosolic viral RNA (1). The membrane-bound TLRs recognize endosomally localized RNA and DNA containing CpG sequence motifs (2).

With respect to microbial non-CpG DNA, which has long been proposed to act as a PAMP, the situation is less clear. It has been shown that transfected non-CpG DNA or DNA viruses such as modified vaccinia virus Ankara (MVA) strain can trigger production of IFNs via a pathway involving IFN regulatory factor-3 (IRF-3) and the recently described protein stimulator of IFN genes (STING, also called MITA) (3–6). DAI/ZBP-1 has been proposed as the cytosolic receptor for B-form DNA that triggers the IFN system (7). However, subsequent work has seen redundancy for DAI in the system recognizing transfected DNA, and it was shown that DAI is not involved in the innate immune response to DNA vaccines in vivo (8, 9). Two groups recently showed that in some instances the RNA polymerase III can act as a sensor of foreign DNA that indirectly activates an IFN response by transcribing the invading DNA into double-stranded 5'-triphosphate modified RNA intermediates that act as ligands for the cytoplasmic RNA sensor RIG-I (10, 11). Apoptotic responses after exposure of cells to immunostimulatory DNA have not been detailed in any of the studies mentioned above.

Recently, several groups have described a member of the HIN200 gene family, AIM2, to be involved in the inflammatory response to cytosolic DNA and DNA viruses in mouse macrophages and PMA-treated THP-1 cells (12–16). AIM2 directly recognizes DNA via its HIN domain and binds the adaptor protein apoptosis-associated speck-like protein containing a caspase activating and recruitment domain (ASC) via its pyrin domain,

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The online version of this article contains supplemental material.

Abbreviations used in this article: AIM2, absent in melanoma 2; APAF-1, apoptotic protease activating factor-1; ASC, apoptosis-associated speck-like protein containing a caspase activating and recruitment domain; ATM, ataxia telangiectasia mutated; ATR, ATM-related; BAX, BCL2-associated X protein; Bcl2L12, Bcl2-like 12; IFNAR, IFN type I receptor; IRF-3, IFN regulatory factor-3; IP-10, IFN-inducible protein-10; MOI, multiplicity of infection; MVA, modified vaccinia virus Ankara strain; PAMP, pathogen-associated molecular pattern; PI, propidium iodide; RIG-I, retinoid acid-induced gene I; siRNA, small interfering RNA.

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leading to the formation of an inflammasome containing active caspase-1. As a consequence, cells produce and secrete mature IL-1 $\beta$ . Some of these studies noted that cells undergo cell death after transfection of DNA in an AIM2 and ASC-dependent manner. Roberts et al. (12) show that DNA electroporated into murine macrophages activates caspase-3. However, the study by Fernandes-Alnemri et al. (14) qualifies the cell death as pyroptosis, a rather recently described type of regulated cell death mediated by caspase-1 activation that has so far been thought to be caspase-3 independent. Hornung et al. (13) show that murine macrophages lose cell viability after either transfection with poly(dAdT) or infection with MVA and that this requires the DNA receptor AIM2. Thus, it seems to be clear that cytosolically delivered DNA introduced either by lipofection or by infection with MVA acts as an important PAMP and can lead to AIM2-dependent cell death in macrophages. However, which mode(s) of regulated cell death are used by macrophages remains to be clearly demonstrated. Importantly, it is also not clear whether cell death induction after detection of microbial DNA is restricted to macrophages or represents a general phenomenon observed also in other cell types.

## Materials and Methods

### Reagents

Poly d(AT:TA), a double-stranded heteropolymer with dA and dT as alternating sequence, and poly (dA:dT), a double-stranded homopolymer with poly dA annealed to poly dT, staurosporine (used at 2  $\mu$ M), and caffeine were purchased from Sigma-Aldrich. Chemically synthesized small interfering RNAs (siRNAs) were purchased from Eurofins MWG Operon (for siRNA sequences, see Supplemental Table I). RT-PCR primers were purchased from Metabion. MVA wild-type virus, its F1L deletion variant, and isolated viral DNA were propagated and generated as described previously (17). Anti-caspase-3, anti-caspase-8 (1C12), anti-caspase-9, anti-p53, and phosphospecific Abs to serines 15 and 46 of p53 were obtained from Cell Signaling Technology (Danvers, CA), and anti- $\beta$ -actin was from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary Abs were from Promega (Madison, WI). zVAD-fmk was from Bachem (Torrance, CA), etoposide, and the polymerase III inhibitor *N*-[1-(3-(5-chloro-3-methylbenzo[*b*]thiophen-2-yl-1-methyl-1*H*-pyrazol-5-yl)]-2-chlorobenzenesulfonamide were from Calbiochem (San Diego, CA).

### Cell culture

Human HEK293, HUH7.0, and H1339 cell lines were grown in DMEM (PAA Laboratories, Coelbe, Germany) supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. Human THP-1 cells were maintained in very low endotoxin RPMI 1640 medium (Biochrom AG) supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. 1205 Lu cells were maintained in a culture medium consisting of MCD153 (Sigma-Aldrich) with 20% Leibovitz's L-15 (PAA Laboratories), 2% FBS (PAA Laboratories), 1.68 mM CaCl<sub>2</sub> (Sigma-Aldrich), and 5  $\mu$ g/ml insulin (Sigma-Aldrich). PBMC, monocytes, and monocyte-derived dendritic cells from healthy donors were generated as described previously (18).

### Quantification of cell viability

Viable cells were quantified using a fluorimetric assay (CellTiter-Blue Cell Viability Assay; Promega, Mannheim, Germany), according to the manufacturer's protocol. Viable cells with intact metabolism are determined by their ability to reduce cell-permeable resazurin to fluorescent resorufin. After 1 h of incubation at 37°C, fluorescence was measured using a Mithras LB 940 multimode microplate reader (Berthold Technologies, Bad Wildbad, Germany). Results are given as percent viability whereby cells treated with the mock control (Lipofectamine complexed with a nonstimulatory oligonucleotide) are set to 100%.

### Quantification of apoptotic cells and cell death

Cells were analyzed by staining with FITC-labeled annexin V (Roche) and propidium iodide (PI) (Sigma-Aldrich) as described previously (19). Annexin-FITC<sup>+</sup>/PI<sup>-</sup> and annexin-FITC<sup>+</sup>/PI<sup>+</sup> cells are grouped together and displayed as percent annexin V-positive cells of all analyzed cells. In experiments comparing the effects of different siRNA-mediated gene knock-

downs, results are given as  $\Delta$  annexin positive (%) describing the difference in percentage of annexin-positive cells of samples stimulated with DNA or virus minus their mock control for each gene knockdown condition.

### ELISA

Human IFN-inducible protein-10 (IP-10) and IL-1 $\beta$  was measured in culture supernatants using ELISA sets (BD Biosciences), according to the manufacturer's instructions. Human phospho-ataxia telangiectasia mutated (ATM) (serine 1981) was measured in lysates of  $6 \times 10^5$  1205 Lu cells using an ELISA set, according to the manufacturer's instructions (DYC 1655-2; R&D Systems).

### RT-PCR

Total cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche), according to the manufacturer's protocol. RNA was reverse transcribed using oligo-dT primers, and mRNA levels were analyzed by RT-PCR (Roche Light Cycler LC480 II) using gene-specific primers and probes from the Roche universal probe library. The following primer and probe combinations were used: AIM2 (primer 1, 5'-CACCAAAAGTCTCTCCTCATGTT-3'; primer 2, 5'-AAACCCCTTCTCTGATAGATTCTCTG-3'; probe number 49); apoptotic protease activating factor-1 (APAF-1) (primer 1, 5'-CTGCTGATGGTGCAAGGATA-3'; primer 2, 5'-CCAATAGGCCACTAGTATGAATGTC-3'; probe number 87); ASC (primer 1, 5'-CTCACCGCTAACGTGCTG-3'; primer 2, 5'-CCGGTGCTGGTCTATAAAGTG-3'; probe number 81); ATM (primer 1, 5'-TGATAGTAGTGTTAGTGATGCAACG-3'; primer 2, 5'-CAGCTAAAGGATTAATGGCACCT-3'; probe number 80); ATM-related (ATR) (primer 1, 5'-CGCTGAAC-TGTACGTGGAAA-3'; primer 2, 5'-CAATTAGTGCCTGGTGAACATC-3'; probe number 21); caspase-1 (primer 1, 5'-ATGCCTGTTCTGTGATGTG-3'; primer 2, 5'-CTCTTTTCAGTGGTGGGCATC-3'; probe number 71); DAI (primer 1, 5'-GAGGAGTCGACGGGTCTG-3'; primer 2, 5'-GACTTCTGGATTGTGTGTCTGC-3'; probe number 36); E3L (primer 1, 5'-TTTCGCGGGATACATCAT-3'; primer 2, 5'-GACAACGGAGGCGG-ATAA-3'; probe number 89); IFN type I receptor (IFNAR) (primer 1, 5'-TTACACCATTTCGCAAAGCTC-3'; primer 2, 5'-TGTTCCAGGAGAG-ATGTGTATCA-3'; probe number 65); IRF-3 (primer 1, 5'-CTTGGAAGCAGGCGCTAC-3'; primer 2, 5'-ATCCCTCCCGGAACATA-3'; probe number 18); p53 (primer 1, 5'-CTTCCACGACGGTGACA-3'; primer 2, 5'-TCCTCCATGGCAGTGACC-3'; probe number 71); STING (primer 1, 5'-GATATCTGCGGCTGATCCTG-3'; primer 2, 5'-GCAGGTTGTTGTA-ATGCTGATT-3'; probe number 1); RIG-I (primer 1, 5'-TGGACCCCTA-CCTACATCTGA-3'; primer 2, 5'-GGCCCTTGTGTTTCTCA-3'; probe number 69); and MAVS (primer 1, 5'-TGTCTGCCAGCACAGTC-C-3'; primer 2, 5'-GAGCTGCTGGGTCTCTCT-3'; probe number 32).

### Cell stimulation and siRNA experiments

If not stated otherwise cell lines were stimulated by transfection of DNA preparations at a concentration of 4  $\mu$ g/ml for 18 h using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's manual. For mock stimulation, the highest amount of transfection reagent/oligonucleotide applied in that particular experiment was matched as control. Infection by MVA was done at a multiplicity of infection (MOI) of three if not stated otherwise for 18 h. In cases where irradiated virus was used, it was subjected to UVA/B treatment in a 6-well plate on ice for 30 min at a distance of 1 cm with an LKB 2011 Macrovue transilluminator that had a peak output at 302 nm. Successful irradiation was confirmed by loss of early gene transcription. For knockdown experiments, 1205 Lu cells were seeded in 24-well plates at a density of  $3 \times 10^4$  cells/well and allowed to adhere overnight. On the next day, cells were transfected with siRNA oligonucleotides (50 nM) for 48 h using Lipofectamine RNAiMAX. Cells were again transfected with siRNA (50 nM) and simultaneously stimulated with poly d(AT:TA) (4  $\mu$ g/ml) or MVA (3 MOI) for 18 h. Culture supernatants were collected for ELISA analysis before measuring cell viability. Afterward, cells were harvested and processed for Western blot, RT-PCR, or FACS analysis, respectively. For siRNA experiments in THP-1, cells were seeded in 24-well plates at a density of  $3 \times 10^4$  cells/well and differentiated with PMA (1  $\mu$ M) overnight. Before PMA treatment, siRNA (50 nM) was transfected using DreamFect, according to the manufacturer's recommendations. After 24 h, cells were again transfected with siRNA and stimulated with poly d(AT:TA) (4  $\mu$ g/ml) using RNAiMAX or MVA (3 MOI) for 18 h prior to analysis.

### Statistical analysis

For statistical analysis, two-tailed Student *t* test was used to assess the significance of mean differences. Differences were considered significant at a  $p \leq 0.05$ . Statistical significance is indicated by ns for  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . Data are displayed as means  $\pm$  SEM.



## Results

### *Nonmyeloid cells die in response to DNA challenge and DNA virus infection in the absence of a functional AIM2 inflammasome*

To analyze the effect of cytosolic foreign DNA delivery on cell survival, we started by screening a variety of human cell lines derived from different tissues. HEK293 (embryonic kidney cell line), 1205 Lu (melanoma cell line), and HUH7 (hepatoma cell line) showed signs of cell death in response to transfection with increasing amounts of dsDNAs reaching >50% dead or dying cells. Cell death was measured using a metabolic viability assay and exposure of phosphatidyl serine residues on the cell surface detected by flow cytometry after staining with FITC-labeled annexin V (Fig. 1A, 1C, Supplemental Fig. 1A–C). As described for the IFN response triggered by transfected DNA (6), cell death responses could be induced by a variety of different DNAs like the synthetic double-stranded model DNAs poly d(AT:TA), poly (dA:dT), or isolated plasmid DNA (Fig. 1A, Supplemental Fig. 1A–C). Of note, transfection of viral DNA isolated from MVA showed the same effects as the model DNAs (Fig. 1B, 1C). In all cases, cell death induction critically depended on transfection of the DNA, because adding uncomplexed DNA to the culture medium did not induce cell death (Fig. 1B, 1C, Supplemental Fig. 1C). As described for the IFN response (6), transfection of single-stranded DNA like poly A does not induce cell death in this setting (data not shown). Taken together, these results indicate that cells of nonmyeloid origin die after cytosolic delivery of viral and synthetic foreign dsDNAs similar to what has been shown for macrophages.

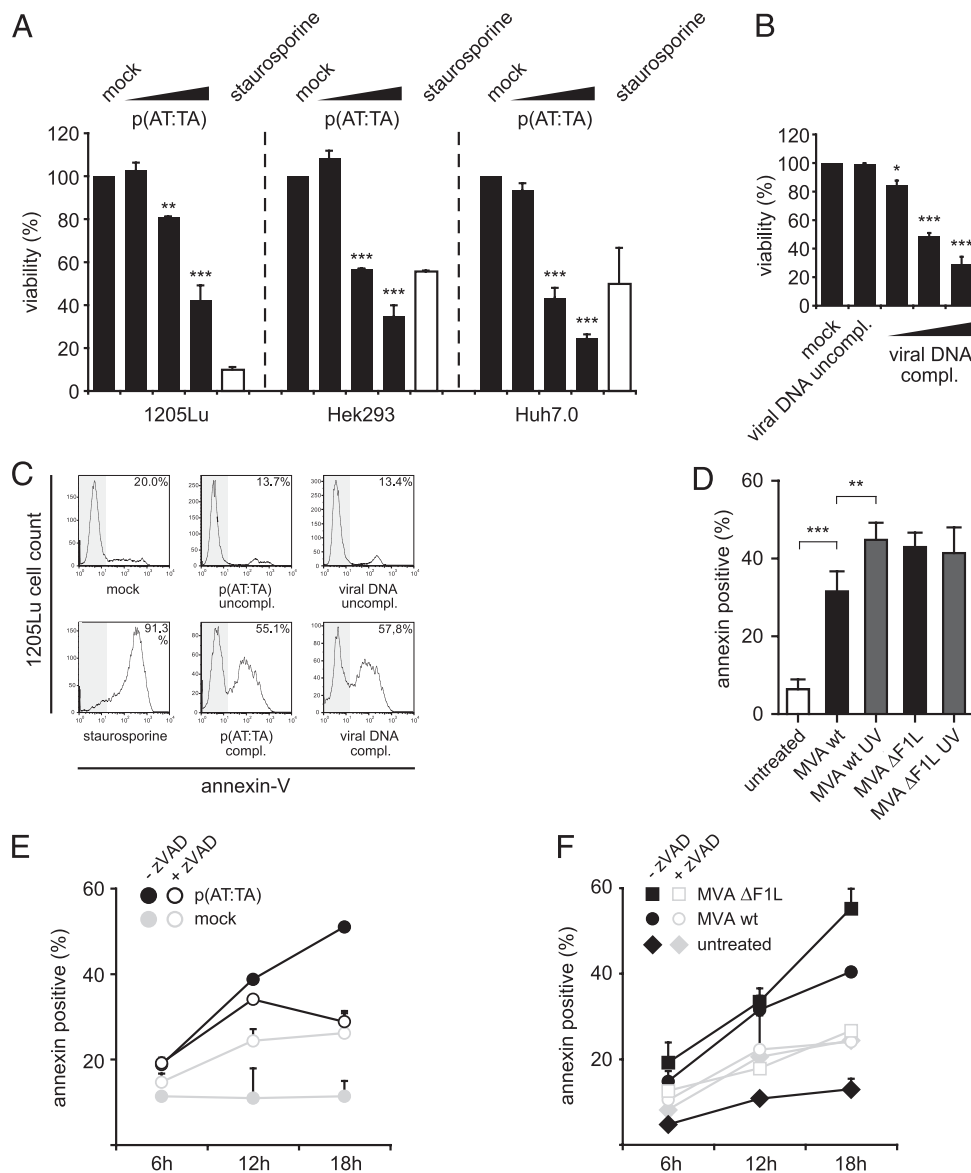
MVA is a highly attenuated derivative of the DNA virus vaccinia that is widely used as a vehicle for vaccination. MVA was generated by serial passage in chicken embryo fibroblast cultures (20). Compared with conventional vaccinia virus strains, the MVA genome contains large deletions and mutations (21, 22). As a consequence, MVA is unable to produce new infectious progeny in human and many other mammalian cells. Importantly, however, in nonpermissive cells, the intracellular molecular biology of MVA infection remains nevertheless unimpaired, supporting viral DNA synthesis and expression of all classes of viral genes in the cytoplasm (23). When we infected melanoma cells with wild-type MVA or a mutant lacking the viral BCL-2 functional homolog FIL ( $\Delta$ FIL), we observed up to 40% more cell death than in untreated cells (Fig. 1D, Supplemental Fig. 1D) (17, 24). Interestingly, mutant MVA was always slightly more efficient in killing cells than the wild-type virus (Figs. 1D, 1F, 3B, Supplemental Fig. 1D). To check whether this cell death induction required viral transcription, replication, or newly synthesized viral proteins, we also infected cells with UV-irradiated viral preparations in the same assays (Fig. 1D). The complete absence of MVA early gene transcription confirmed successful inactivation of the viral genome as a template (Supplemental Fig. 2A). Irradiated MVA preparations induced cell death to a similar degree as untreated viruses (Fig. 1D). Wild-type MVA even showed a somewhat higher ability to induce cell death when irradiated. This could be due to the inhibited transcription of the antiapoptotic viral gene F11, because this UV irradiation-induced difference was not seen for the mutant lacking the BCL-2 functional homolog FIL. These results show that infection of cells with MVA induces cell death independently of viral transcription or processes that happen downstream. As expected, the same viral preparations and synthetic DNAs also induced cell death in THP-1 cells that are of myeloid origin. This induction was enhanced by prestimulating the cells with the phorbol ester PMA (Supplemental Fig. 1D). Taken together with the already published results on AIM2-dependent recognition of MVA in macrophages,

the data are consistent with a model in which introduction of viral or synthetic DNA by either transfection or infection can induce cell death in cells of both myeloid and nonmyeloid origin.

To investigate the timing of cell death and to collect more basic information on what type of cell death is observed in nonmyeloid cells, we stimulated 1205 Lu cells with poly d(AT:TA) or infected them with wild-type or mutant MVA in presence or absence of the pan-caspase inhibitor zVAD-fmk. We observed that after 6 h, cells showed signs of apoptosis after DNA challenge or MVA infection increasing over time. We noted that administration of zVAD-fmk was able to inhibit cell death induction, indicating that it largely depended on the activity of caspases (Fig. 1E, 1F, Supplemental Fig. 1E). Importantly, we found DNA-induced cell death in 1205 Lu melanoma cells to be independent of secondary soluble factors, because the supernatant of cells transfected with DNA did not affect previously untreated cells even after long periods of incubation (Supplemental Fig. 2B). Taken together, these observations suggested that the cell death we observe is a caspase-dependent cell death that is induced cell autonomously.

Because the DNA receptor AIM2 was recently described as the mediator of cell death in response to DNA transfection and MVA infection in murine macrophages, we were interested if it is also expressed in our cellular systems. To check this, different primary myeloid cells and cell lines derived from different tissues were left untreated or stimulated with IFN- $\alpha$  and analyzed for the basal and induced expression of the inflammasome components AIM2, ASC, and caspase-1 by quantitative real-time PCR. Interestingly, basal mRNA levels of AIM2, ASC, and caspase-1 were much higher in cell preparations containing myeloid cells (human PBMCs, monocytes, monocyte-derived dendritic cells, and the THP-1 monocytic leukemia cell line) compared with cells of nonmyeloid origin (1205 Lu melanoma cells, H1339 lung epithelial cells, Jurkat T cells, Huh7.0 hepatoma cells, and HEK293 embryonic kidney cells) in which AIM2 mRNA was hardly or not at all detectable, and ASC and caspase-1 were absent (Fig. 2). Stimulation of myeloid cells with IFN- $\alpha$  increased AIM2 levels significantly ( $p < 0.01$  for differences between IFN-stimulated and nonstimulated conditions), whereas no effect or even a slight decrease of AIM2 expression could be observed upon treatment of nonmyeloid cells (Fig. 2A). The influence of IFN on ASC and caspase-1 expression was less pronounced and consistent (Fig. 2B, 2C). These results show that the components of the AIM2 inflammasome are differentially expressed, with high expression of all necessary components primarily in cells of myeloid origin. They are also consistent with the earlier observation that epithelial cells such as HEK293 only produce mature IL-1 $\beta$  after combined transfection of AIM2, ASC, and caspase-1 (13).

To further test the possibility that the AIM2 inflammasome, despite low expression levels, could affect DNA-induced cell death in nonmyeloid cells, we depleted residual AIM2 from 1205 Lu melanoma cells using siRNA (for knockdown efficiency, see Supplemental Fig. 3E) and compared the effect to the knockdown of AIM2 in THP-1 cells. Depletion of AIM2 showed no effect on cell viability or activation of caspase-3 induced by poly d(AT:TA), MVA wild-type, or MVA  $\Delta$ FIL in 1205 Lu cells (Fig. 3A, 3B). In THP-1 cells, however, the same siRNA targeting AIM2 reduced IL-1 $\beta$  secretion to background levels and significantly reduced cell death after stimulation with poly d(AT:TA) (Fig. 3C, 3E), thereby confirming the published role of AIM2 for myeloid cells (12–15). Of note, when we used MVA  $\Delta$ FIL, increased cell death compared with wild-type virus was not only seen in 1205 Lu cells but also in the monocyte-derived cell line THP-1 (Fig. 3D, Supplemental Fig. 1D). Because the FIL protein specifically antagonizes mitochondrial apoptosis, this suggested that mitochondrial apoptosis might



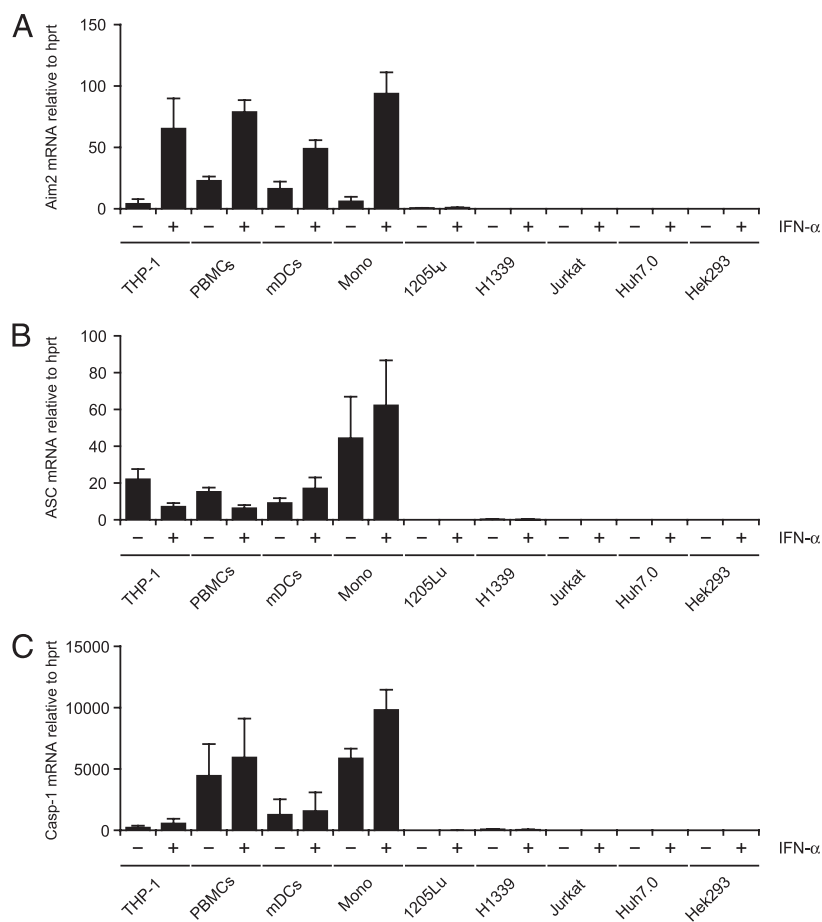
**FIGURE 1.** Human myeloid and nonmyeloid cells die upon challenge with DNA. *A*, 1205 Lu human melanoma cells as well as HEK 293 and Huh 7 cells were treated with transfection reagent complexed with a nonstimulatory oligonucleotide (mock), staurosporine (2  $\mu$ M), or transfected with increasing amounts (1, 4, and 8  $\mu$ g/ml) of poly d(AT:TA) complexed with transfection reagent. Viability of the cells was determined after 18 h. *B*, 1205 Lu cells were mock treated or treated with MVA DNA in the presence (1, 4, and 8  $\mu$ g/ml, viral DNA compl.) or absence (8  $\mu$ g/ml, viral DNA uncompl.) of transfection reagent as above and assayed for cell viability after 18 h. *C*, 1205 Lu cells were mock treated, stimulated with poly d(AT:TA) alone (uncompl.), and poly d(AT:TA) complexed with transfection reagent or viral DNA isolated from MVA with or without transfection reagent. Cell death induction after 18 h was determined by FACS staining of annexin V. *D*, 1205 Lu cells were infected with wild-type MVA and a mutant lacking the F1L gene ( $\Delta$ F1L) that had been left untreated or had been irradiated with ultraviolet light (UV). Cell death induction after 18 h was determined by staining for annexin V binding and analysis by flow cytometry. *E* and *F*, 1205 Lu cells were stimulated in the presence or absence of 30 min preincubation with zVAD-fmk (50  $\mu$ M) with the indicated stimuli. Annexin V positivity was determined 6, 12, and 18 h after stimulation by flow cytometry. Data show means  $\pm$  SEM of  $n = 3$  (*B*),  $n = 4$  (*A*), and  $n = 6$  (*D*) independent experiments. *D*, *E* (mean  $\pm$  SEM of duplicates), and *C* show data of one of three representative experiment with similar results.

play a role in MVA-induced cell death also in myeloid cells. Taken together, these data show that cell death triggered by cytosolic delivery of DNA is a response that can be observed in a variety of nonmyeloid cells, despite their apparent lack of a functional AIM2 inflammasome, indicating that DNA recognition can trigger cell death by an additional AIM2-independent pathway.

#### *Cell death in response to cytosolic DNA delivery is independent of the pathways leading to IFN type I*

It has been shown that transfection of various DNAs including poly d(AT:TA) triggers the IFN system as one arm of the antimicrobial defense (4, 6, 25). To check whether IFN signaling and cell death

induction are linked, we examined the possibility that IFN signaling is a prerequisite for the cell death observed in this study. To this end, we treated 1205 Lu cells with siRNAs targeting the transcription factor IRF-3 or the IFNAR (for knockdown efficiency, see Supplemental Fig. 3A, 3B) and analyzed production of the IFN-inducible protein-10 (IP-10) and cell death induction after stimulation with poly d(AT:TA) or infection with MVA. Whereas IP-10 production in response to stimulation with poly d(AT:TA) was abolished in cells depleted of IRF-3 or IFNAR but not in control siRNA-treated cells (Fig. 4B), cell death responses stayed almost unaffected (Fig. 4A, 4C). In line with these results, we did not observe reduced cell death after DNA transfection when we



**FIGURE 2.** Differential expression of the AIM2 inflammasome components. Human PBMCs, isolated monocytes, and monocyte-derived dendritic cells from four different healthy donors as well as different cell lines (THP-1 monocytic leukemia cells, 1205 Lu melanoma cells, H1339 lung epithelial cells, Jurkat T-cells, Huh 7 hepatoma cells, and HEK293 embryonic kidney cells) were left untreated or stimulated with rIFN- $\alpha$  (1000 U/ml) for 4 h. RNA preparations of these cells were subsequently analyzed by quantitative RT-PCR for expression levels of AIM2 (A), ASC (B), and caspase-1 (C). The data are expressed as copies relative to 100 copies of the housekeeping gene HPRT and represent means + SEM of four different donors for primary cells and three independently tested batches for each cell line, respectively.

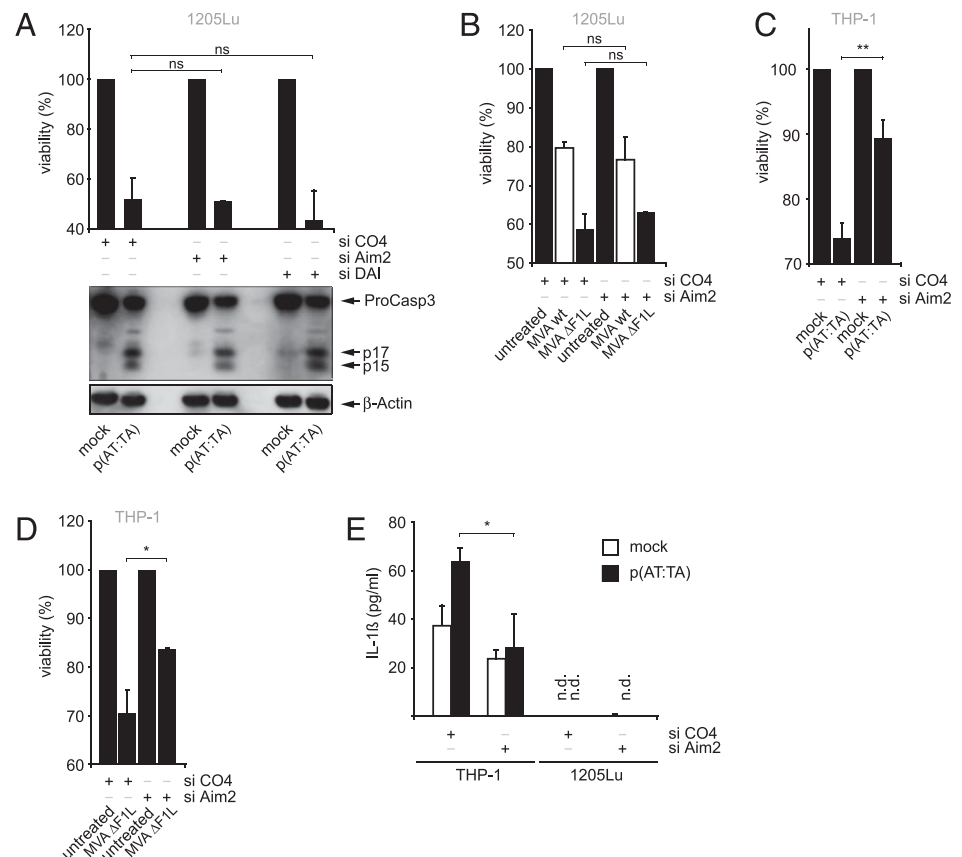
depleted the potential cytosolic DNA receptor DAI/ZBP-1 (Fig. 3A, Supplemental Fig. 3F) or STING/MITA (Supplemental Fig. 3C, 3D), which has been described to be required for IFN signaling in response to immunostimulatory DNA transfection (5, 7). The recently described induction of IFN by poly d(AT:TA) via RNA polymerase III-dependent transcription into 5'-triphosphate RNA and detection via RIG-I and its MAVS-dependent signaling (10, 11) opens the possibility that poly d(AT:TA) and MVA infection could also trigger apoptosis via this pathway. However, although blocking RNA polymerase III with a small molecule inhibitor almost completely abolished the IP-10 induction by poly d(AT:TA) (Fig. 4E), cell death induction by poly d(AT:TA) and infection with MVA was only affected minimally (Fig. 4D). The small decrease in cell death induction in the presence of the polymerase III inhibitor—even though consistent—argues that, if at all, the polymerase III-dependent pathway can account for only a small part of the effect, whereas most of it is mediated by a different pathway. In line with this notion, depleting MAVS and RIG-I via siRNA abolished the induction of IP-10 by poly d(AT:TA), but left the cell death induction via MVA and poly d(AT:TA) in 1205 Lu cells intact (Supplemental Fig. 2C, 2D; for knockdown efficiency, see Supplemental Fig. 3J, 3K). Taken together, these data suggest that invading DNA induces cell death and IFN via two different and largely independent pathways in our setting.

#### *Cytoplasmic delivery of microbial DNA triggers the mitochondrial apoptosis pathway via DNA damage signaling proteins*

Caspase dependency, caspase-3 activation, and flipping of membrane lipids are hallmarks of apoptotic cell death. Apoptosis is

classically divided into the extrinsic pathways mediated by caspase-8 and the intrinsic pathway, in which the activation of proapoptotic BCL-2-family members BAX and BAK leads to mitochondrial outer membrane permeabilization and subsequently to the formation of an apoptosome containing cytochrome *c*, Apaf-1, and caspase-9. The observation that in all our cellular infection systems a mutant virus lacking a BCL-2 homolog was more efficient in inducing cell death already indicated that the mitochondrial pathway might play an important role. To further investigate which pathway is involved in killing nonmyeloid cells stimulated with DNA, we analyzed cells depleted of caspase-8 or caspase-9 with respect to cell viability and activation of these two caspases after stimulation. Whereas cells in which caspase-8 was knocked down by RNA interference still died and activated caspase-9 after cytosolic delivery of DNA, cells depleted of caspase-9 remained significantly more viable compared with controls even though caspase-8 cleavage could be observed under these conditions (Fig. 5A). This indicated that caspase-9 plays a major role in mediating the cell death observed in these cells and that potential cross-talk via caspase-8 cannot compensate. Consistent with a role of mitochondrial apoptosis, we found the proapoptotic BCL-2 family member BAX and the apoptosome component Apaf-1 to be required for DNA-induced caspase-3 activation and cell death (Figs. 5B, 5C, 4A, 4C, Supplemental Fig. 3G for knockdown efficiencies). This is also in line with our initial observation that secreted factors in the medium do not control the apoptosis we observe in this study, as one might have expected in TNFR-family-mediated apoptosis. Supporting the physiological relevance of these observations, we found that BAX and Apaf-1 RNA interference also strongly reduced cell death postinfection with wild-type or FIL-deleted MVA (Fig. 5D, 5E). Taken to-

**FIGURE 3.** Cell death of nonmyeloid cells in response to DNA and vaccinia virus infection does not require AIM2 or DAI. **A**, 1205 Lu cells were transfected with siRNAs targeting AIM2, DAI, or a nonsilencing control (CO4), respectively, and left unstimulated or stimulated with poly d(AT:TA). Cell viability was analyzed after 18 h, and caspase-3 activation was examined by Western blot analysis. **B**, Control-treated or AIM2-depleted 1205 Lu cells were left untreated or infected with wild-type MVA or a mutant lacking the FIL gene ( $\Delta$ FIL) and were assayed for cell viability after 18 h. **C** and **D**, Control-treated or AIM2-depleted THP-1 cells were mock treated, stimulated with poly d(AT:TA) (**C**), or infected with mutant MVA (**D**) and assayed for cell viability after 18 h. **E**, IL-1 $\beta$  was measured in the supernatant of THP-1 and 1205 Lu cells that were treated as in **A** or **C**, respectively. Data show means  $\pm$  SEM of  $n = 4$  (**A**, **B**, **C**, **E**) or  $n = 3$  (**D**) independent experiments.



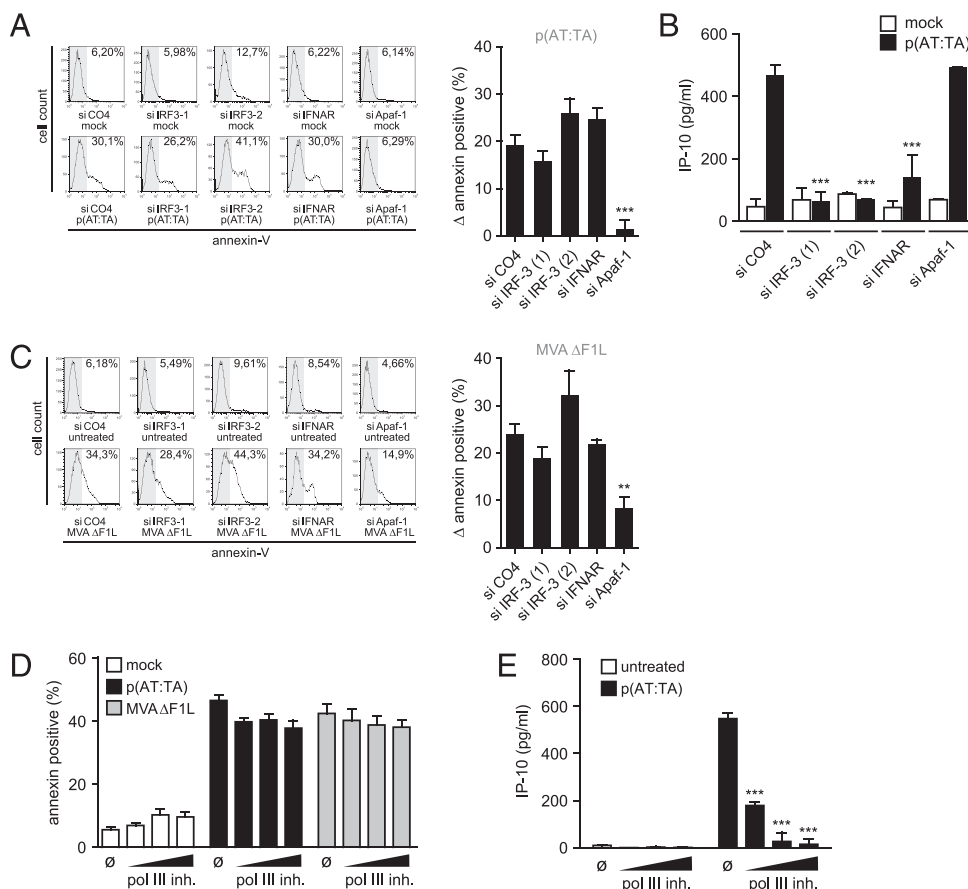
gether, these results demonstrate that cell death in nonmyeloid cells after challenge with transfected DNA or DNA virus infection primarily occurs via a mitochondrial apoptosis pathway.

Next, we were interested in the pathways that trigger mitochondrial apoptosis upon DNA delivery. It is known that DNA viruses such as EBV and adenoviruses can activate or even exploit p53-dependent DNA damage signaling pathways. However, these viruses deliver their DNA into the nucleus bypassing the cytoplasm. MVA is known to deliver its DNA exclusively to the cytoplasm (26), and the role for p53 upon MVA infection is unclear. Likewise, a role for p53 following cytosolic delivery of immunostimulatory DNA has not been examined. Thus, we investigated p53 activation and the consequences of p53 knockdown upon stimulation with foreign DNA and MVA infection. Indeed, stimulation with DNA or infection with different MVA preparations led to induction of p53 and phosphorylation at serines 15 and 46 (Fig. 6A). These phosphorylation sites have been implicated as target sites of the ATM pathway (serine 15) and are required for p53-mediated apoptosis (serine 46) (27). Consistently, depletion of p53 by siRNA greatly reduced apoptosis in response to transfection of poly d(AT:TA) or infection with MVA. p53 depletion was thereby as effective as the depletion of apaf-1, and this effect was sustained over a period of  $>36$  h after stimulation (Figs. 6B–E, 7F, 7G; for knockdown efficiency, see Supplemental Fig. 3L). Taken together, these results strongly indicate that DNA stimulation and infection with MVA trigger p53 phosphorylation and subsequently p53-dependent apoptosis in cells.

In response to DNA damage or replication stress, mammalian cells activate signal transduction pathways leading to repair, activation of cell cycle checkpoints, gene silencing, or, if the damage is irreparable, induction of apoptosis. ATM/ATR (ataxia telangiectasia and Rad3-related) kinases are important mediators in

these signaling pathways that couple DNA damage to cell cycle arrest and apoptosis (28). To investigate whether the ATM pathway could be involved in the p53-dependent apoptosis, we checked whether ATM is activated by either DNA stimulation or MVA infection. We observed autophosphorylation of ATM on serine 1981 from 1.5 h after stimulation when we transfected cells with poly d(AT:TA) or infected them with MVA (Fig. 7A, 7B). To check whether the activity of ATM plays a role in DNA-induced or DNA virus-induced cell death, first, we resorted to inhibiting the ATM/ATR-family kinases with caffeine (29). Whereas cells treated with poly d(AT:TA) or infected with MVA showed normal induction of apoptosis, cells pretreated with caffeine demonstrated a strongly reduced induction of cell death (Fig. 7C, 7D). These data suggested that cell death induced by vaccinia virus infection or poly d(AT:TA) in nonmyeloid cells involves kinases that are commonly associated with DNA damage signaling. Consistently, we observed a loss of p53 phosphorylation after treatment of DNA-stimulated cells with caffeine (Fig. 7E). To test whether this effect was a specific result of inhibition of ATM and ATR, we knocked down their expression by siRNA (for knockdown efficiencies, see Supplemental Fig. 3H, 3I). We found that ATM and, to a lesser degree, ATR expression were required for DNA-induced and MVA-induced apoptosis similarly to Apaf-1 and p53 (Fig. 7F, 7G). Importantly, the requirement was observed for both the untreated and the irradiated preparations of MVA, indicating that irradiation did not prevent detection and did not cause aberrations that induce detection (Fig. 7G). In summary, these results clearly show that intracellular foreign DNA and infection with a DNA virus that delivers its DNA to the cytoplasm activate proteins of the DNA damage signaling pathway that are then required to induce intrinsic apoptosis in nonmyeloid cells. They suggest a model in which recognition of foreign DNA in non-





**FIGURE 4.** Cell death in response to DNA is independent of the pathways leading to IFN type I. A–C, 1205 Lu cells were transfected with a nonsilencing control siRNA (CO4) or siRNAs targeting IRF3, IFNAR, or Apaf-1 and were mock treated, stimulated with p(AT:TA) (A, B), or infected with the F1L-deficient MVA mutant (C). Eighteen hours after stimulation, cells were assayed for annexin binding by flow cytometry (A, C), and IP-10 levels in the cell culture supernatant were measured by ELISA (B). D, E, 1205 Lu cells were preincubated for 30 min with increasing concentrations (0, 10, 20, and 30  $\mu$ M) of an RNA polymerase III inhibitor (pol III inh.) and subsequently stimulated with poly p(AT:TA) or infected with the F1L-deficient MVA mutant. Eighteen hours after stimulation, cells were assayed for annexin binding by flow cytometry (D), and IP-10 levels in the cell culture supernatant were measured by ELISA (E). Data show representative histograms (A, C) and means + SEM of  $n = 3$  (A–C) and  $n = 4$  (D, E) experiments.

myeloid cells leads to the activation of ATM, which in turn phosphorylates p53 leading to induction of mitochondrial apoptosis independently of currently known proteins involved in DNA-induced cytokine production or cell death.

## Discussion

Upon infection with a pathogen harboring DNA as a PAMP, macrophages, but probably even more frequently the nonmyeloid cells lining our body surfaces, will be challenged. In this article, we show that cell lines derived from different nonmyeloid tissues are capable of responding to foreign DNA challenge with the induction of cell death. This response to invading DNA is fundamentally different from the pathway that was recently described for macrophages (12–15) because it depends on p53 activation and the ATM/ATR-family kinases, leads to mitochondrial apoptosis, and is independent of the inflammasome-specific DNA receptor AIM2. AIM2 has been found by unbiased screens and also by its domain composition pointing toward a role in DNA-dependent inflammasome activation. No extensive expression analysis has been done for AIM2 so far, but our data together with the tissue distribution profile published, when AIM2 was first identified (30), makes it very likely that AIM2 is only present and functional in a very limited number of cell types and that the simultaneous expression of ASC, caspase-1, and AIM2—the components required for a functioning AIM2

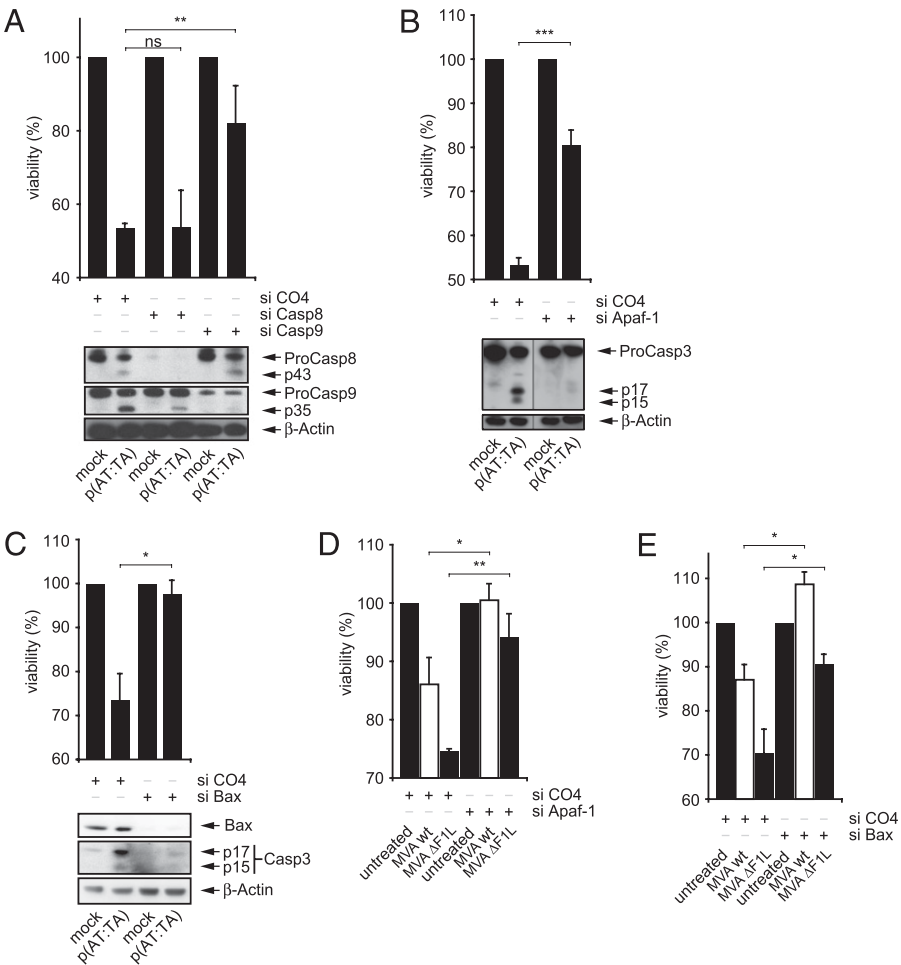
inflammasome—is mainly if not exclusively restricted to myeloid cell types (e.g., monocytes, macrophages, dendritic cells, and granulocytes). Although our data confirm the observation that depletion of AIM2 reduces cell death by DNA transfection in myeloid cells, they indicate that there is an additional system connected to components of the DNA damage response that senses invading DNA. As responses to DNA damage and DNA repair are fundamental and therefore present in every cell type, this second system might be a subversion of classical DNA damage signaling and is likely to be active ubiquitously. Three findings suggest that this system might also be active in myeloid cells, thereby complementing the pyroptotic cell death induced by activation of AIM2: 1) activation of caspase-3 in macrophages (12), which is not part of the pyroptotic pathway; 2) our finding that MVA  $\Delta$ F1L induces more cell death than the wild-type also in THP-1, and 3) only partial inhibition of pyroptosis in macrophages and THP-1 by depletion of AIM2 (14).

Our data indicate that even in cells that have no functional AIM2 inflammasome, cytosolic DNA triggers distinct pathways: one that leads via polymerase III, RIG-I, MAVS, and IRF-3 to the production of IFN and one that is dependent on p53 and independent of polymerase III, RIG-I, and IRF-3 and leads to mitochondrial apoptosis.

In contrast to our results, two recent studies published that apoptosis induced by cytosolic DNA and MVA infection is IRF-3

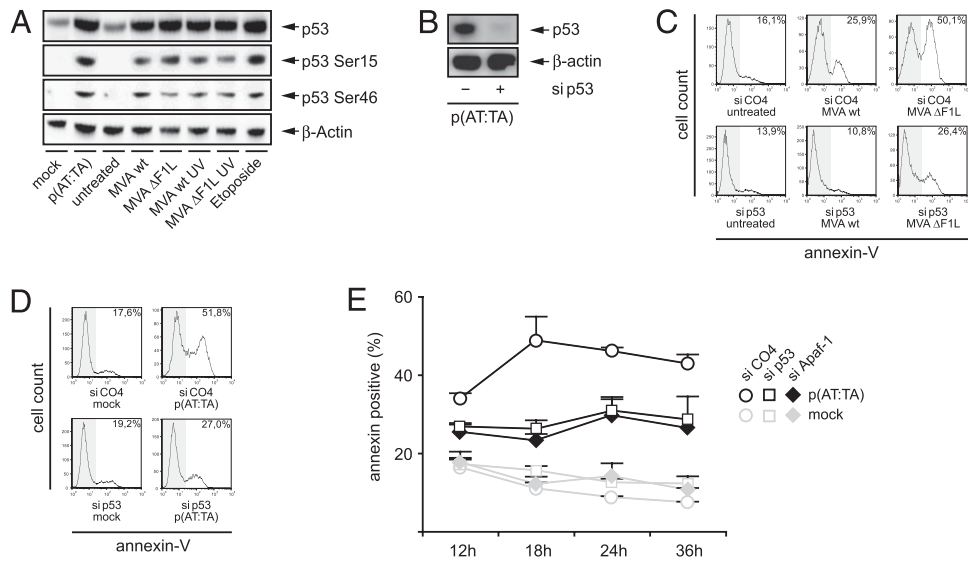


**FIGURE 5.** Nonmyeloid cells die via the mitochondrial apoptosis pathway in response to DNA. *A*, 1205 Lu cells were treated with a nontargeting control RNA (CO4) or siRNAs targeting caspase-8 or caspase-9 and were subsequently mock treated or stimulated with poly d(AT:TA). Cell viability was examined, and cleavage of the caspase-8 and caspase-9 as well as successful knockdown were analyzed by Western blot after 18 h. Actin was used as a loading control. *B*, Control-treated or Apaf-1-depleted cells were stimulated as in *A*. Cell viability was analyzed, and cleavage of caspase-3 was examined by Western blot analysis. *C*, Control-treated or BAX-depleted cells were stimulated as in *A*. Cell viability was analyzed, and activation of caspase-3 and successful knockdown were examined by Western blot analysis. *D*, 1205 Lu cells were either left untreated or infected with wild-type (wt) or FIL-deleted ( $\Delta$ FIL) MVA for 18 h. Prior to infection cells had been treated with control or Apaf-1 siRNA. *E*, The experiment was conducted as in *D* using BAX siRNA. Data shown in the bar diagrams represent means + SEM of  $n = 3$  independent experiments.



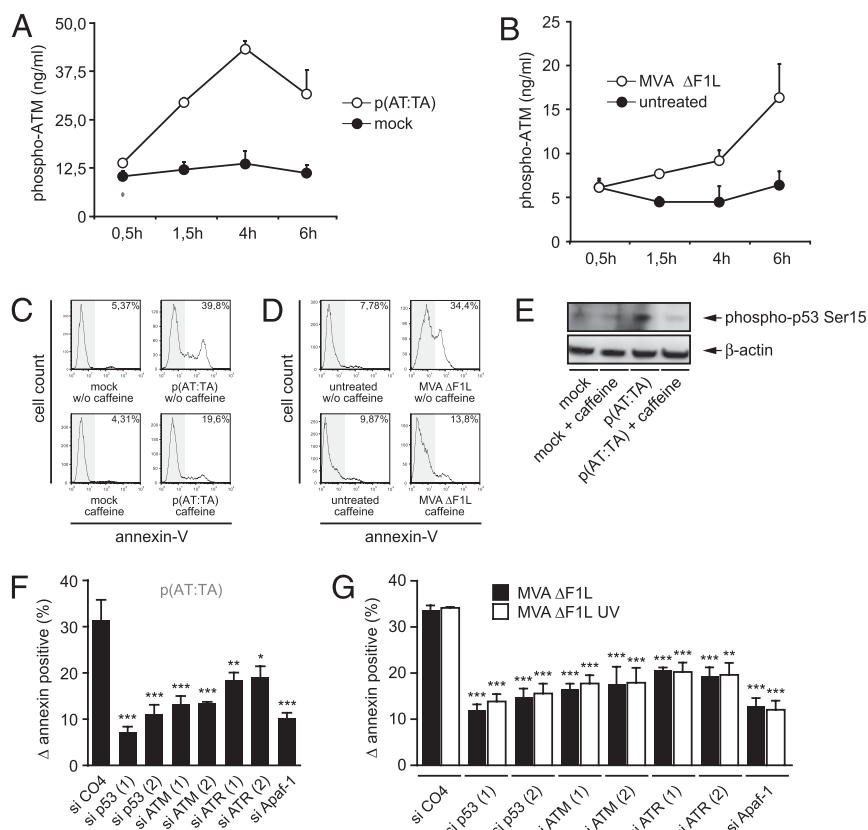
dependent (31, 32). A closer look at these two studies reveals a surprising coincidence and Bcl2-like 12 (Bcl2L12) as a likely explanation for the conflicting data. Chattopadhyay et al. (31)

used a human fibroblast cell line carrying a short hairpin RNA construct targeting human IRF-3 to show the IRF dependence of apoptosis induced by poly d(AT:TA). This short hairpin RNA



**FIGURE 6.** Apoptosis in response to cytosolic DNA and MVA activates and requires p53. *A*, 1205 Lu cells were mock-treated, transfected with poly d(AT:TA), left untreated, or infected with irradiated (UV) or untreated MVA preparations for 4 h as indicated. Cells were then lysed and processed for Western blotting with Abs against total p53, phospho-serine 15 and phospho-serine 46 on p53, and actin as a loading control. Etosiposide (50  $\mu$ M) was used as a positive control for p53 activation. *B*, Knockdown of p53 protein was examined by Western blotting of lysates made from p(AT:TA)-stimulated cells treated with control or p53 siRNA. *C–E*, 1205 Lu cells were treated with control siRNA or the indicated siRNAs targeting p53 and Apaf-1. Cells were then infected with either wild-type or FIL-deleted MVA or stimulated with poly p(AT:TA) and subsequently assayed for signs of apoptosis by FACS. *C*, *D*, Representative histograms at 18 h postinfection and stimulation are shown. *E*, Annexin V binding was measured at the indicated time points after stimulation, and data represent means + SEM of  $n = 2$ .

**FIGURE 7.** DNA damage signaling proteins are required for DNA and MVA-induced apoptosis in nonmyeloid cells. **A**, 1205 Lu cells were mock treated or transfected with poly d(AT:TA) and assayed for autophosphorylation of ATM by ELISA at 0.5, 1.5, 4, and 6 h after stimulation. **B**, 1205 Lu cells that were left untreated or infected with MVA ( $\Delta$ FIL) and assayed as in **A**. **C**, 1205 Lu cells that were left untreated or preincubated with caffeine (5 mM) for 2 h were either mock treated or stimulated with poly d(AT:TA) and then examined for annexin V binding after 18 h. **D**, 1205 Lu cells were left untreated or infected with MVA ( $\Delta$ FIL) in the presence or absence of caffeine and analyzed as in **C**. **E**, 1205 Lu cells that had been mock treated or stimulated with poly d(AT:TA) for 4 h in the presence or absence of caffeine were analyzed for phospho-serine 15 on p53 by Western blot. **F**, **G**, 1205 Lu cells were transfected with the indicated siRNAs and subsequently stimulated with mock or poly d(AT:TA) in **F** or left untreated or infected with irradiated or nonirradiated MVA ( $\Delta$ FIL) in **G**. Annexin V binding was determined by flow cytometry after 18 h. Data in **A** and **B** are shown as means + SEM of duplicates and, like the data in **C–E**, are representative experiments of at least three with similar results. Data in **F** and **G** represent means + SEM of  $n = 3$  independent experiments.



construct targets a sequence in the IRF-3 mRNA (position 3–32 of the mRNA) that is accidentally shared to 100% by the mRNA of the juxtaposed and overlapping Bcl2L12 gene. Ferrer et al. (32) based their conclusion that MVA  $\Delta$ FIL induces apoptosis via IRF-3 largely on the use of cells from IRF-3 knockout mice, which were also used by Chattopadhyay et al. (31). Taniguchi's group, who originally made the IRF-3 knockout mouse in 2000 (33), published in 2009 that by generating this mouse they had accidentally made a double knockout for IRF-3 and Bcl2L12, the gene directly adjacent to IRF-3 (34). In the same article, they showed that cells from this IRF-3<sup>-/-</sup>/Bcl2L12<sup>-/-</sup> mice required re-expression of IRF-3 to rescue them from the defect in dAdT-induced IFN production but needed re-expression of Bcl2L12 to rescue them from the defect in dAdT-induced apoptosis (34). So, unknowingly, it seems in both studies an effect caused—at least in part—by depletion of Bcl2L12 was attributed to IRF-3.

Depending on cell type and experimental setting, pro- and antiapoptotic effects have been described for Bcl2L12, a prolin-rich protein containing a BH2-like domain (34, 35). However, its mode of action is so far incompletely understood, and further studies are required to understand its exact function in the DNA-induced apoptosis pathway. Its known interaction with p53 (35), however, fits well with the p53-dependent apoptosis induced by cytosolic DNA described in this article.

We have published previously that RIG-I and MDA-5 activation induces apoptosis by a pathway being independent of p53 and IRF-3 (19). In the current study, the RLH pathway was clearly activated by poly d(AT:TA) and MVA infection, because the IP-10 production depended on this pathway. However, apoptosis was only slightly reduced by blocking this pathway in our experiments in 1205 Lu cells. In our view, the three pathways AIM2-dependent

pyroptosis, polymerase III/RLH-dependent apoptosis, and the AIM2- and RLH-independent pathway described in this article will synergize and compensate for one another depending on the cell type and stimulus used. An additional source for conflicting results in this context will be the p53 status of cell lines used in studies. Many cell lines like the HeLa cells tested by Ferrer et al. (32) have an inactivated p53 pathway, and therefore, effects of p53-dependent pathways will be masked in such an experimental system.

Viral interaction with DNA damage signaling is a well described phenomenon, especially in the context of viruses that replicate in the nucleus. There is evidence that these viruses activate DNA damage signaling for their own benefit, but there are also examples of viruses expressing proteins that inhibit DNA damage signals (reviewed in Refs. 36 and 37). Our study shows that a DNA damage signaling-like pathway mediates cell death induced by poly d(AT:TA) transfection and infection with MVA, which would rather argue that in this case DNA damage signaling is a defense mechanism. Replication of poxviruses takes place exclusively in the cytosol, and it is thought that vaccinia DNA never enters the nucleus (26). Prototypic DNA damage sensing is assumed to take place in the nucleus, but a very recent study demonstrates that ATM can be found in the cytoplasm and function there (38). Further studies are needed to identify the actual receptor(s) for invading DNA, which trigger this p53-dependent pathway, to identify the location, where invading DNA is recognized, and to determine the features in DNA required to trigger the intracellular sensor besides the fact that the DNA must be double-stranded.

In summary, our study describes triggering of cell death via a DNA damage signaling-like pathway as an additional cellular response besides IFN signaling and activation of the inflammasome that is elicited by invading DNA.

## Disclosures

The authors have no financial conflicts of interest.

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