A novel pathway of cell death in response to cytosolic DNA in *Drosophila* cells

Nazarii Vitak[1], Karyn N. Johnson[2], David P. Sester[1,*], Katryn J. Stacey[1,*]

The University of Queensland, [1]School of Chemistry and Molecular Biosciences, [2]School of Biological Sciences, Brisbane Qld 4072, Australia

Contact

* Corresponding authors:

Dr Katryn Stacey

katryn.stacey@uq.edu.au

Dr David Sester

d.sester@uq.edu.au

The University of Queensland,

School of Chemistry and Molecular Biosciences

Building 76, Cooper Road

St Lucia

Brisbane Qld 4072

Australia

Ph: +61 7 33654640

Fax: +61 7 33654273

Running title: Cell death as a defence against invading DNA

Key Words: Cytosolic DNA, Drosophila, cell death, necrosis, host defence

Abstract

Defence against invading DNA occurs in both mammals and bacteria. Recognition of stray DNA can initiate responses to infection, but may also protect against potentially mutagenic integration of transposons or retrotransposons into the genome. Double stranded DNA (dsDNA) detected in the cytosol of mammalian macrophages can elicit inflammatory cytokines and cell death following assembly of the AIM2 inflammasome. Amongst eukaryotes, responses to cytosolic DNA have so far only been detected in mammals, and AIM2 is mammalian-restricted. In protecting genome integrity, we reasoned that pathways recognising invading DNA should be fundamental to cellular life, and that cell death would be an appropriate response to an overwhelming foreign DNA burden. We found that *Drosophila* S2 cells were killed by transfection of DNA from a range of natural sources. Unlike with mammalian cells, responses were not prevented by DNA denaturation. There was an element of sequence specificity, as synthetic single stranded homopolymers were not toxic, whilst mixed base synthetic DNA caused significant cell death. Death occurred with rapid loss of membrane integrity, and without characteristic features of apoptosis. We have defined a novel defence against invading DNA in *Drosophila.* An active necrotic pathway has not previously been described in insects.

Introduction

Defence against invading DNA occurs in both mammals and bacteria. Bacteria utilise both restriction-modification systems and clustered regularly interspaced short palindromic repeats (CRISPRs) to identify and defend against foreign DNA [1]. In eukaryotes, recognition of stray DNA can initiate responses to infection, but may also protect against potentially mutagenic integration of transposons or retrotransposons into the genome. Eukaryotic responses to cytosolic DNA are thus far only characterised in human and mouse cells. In mammalian cells, double stranded DNA (dsDNA) abnormally located in the cytosol is detected by several receptor systems, leading to type I interferon (IFN), inflammatory cytokines and cell death [2-4]. The receptor for cytosolic DNA eliciting type I IFN production was recently identified as cyclic GMP-AMP synthase (cGAS), which in response to DNA binding catalyses formation of a cyclic dinucleotide from GTP and ATP. This acts as a second messenger and is sensed by STING, which then initiates signalling leading to type I IFN transcription.

A second mammalian response to cytosolic DNA is the assembly of the AIM2 inflammasome, a multiprotein complex that promotes activation of caspase-1 and caspase-8. Caspase-1 cleaves the precursors of IL-1 and IL-18 prior to their release from the cell, and also induces a rapid lytic programmed cell death termed pyroptosis in mammalian macrophages [3-7]. AIM2-activated caspase-8 induces a parallel program of apoptosis [8]. AIM2 is restricted to mammals, and interestingly not even all mammals seem to have AIM2; no gene can be found in bats, and the presence of *AIM2* pseudogenes in elephant, cow, llama, and dog, amongst other species, suggest it has been lost from genomes several times in evolution [9].

Detection of foreign nucleic acid on the basis of unusual structure, sequence or location provides a versatile means of identifying diverse infections, and we would expect this to have been exploited as a strategy by the immune systems of many different species. Cytosolic DNA could be a result not

only of infection, but also of high activity of transposons or retrotransposons, defects in DNA repair, genome instability, or an impaired DNA degradation pathway. In protecting genome integrity, we reasoned that pathways recognising invading DNA should be fundamental to cellular life. Cell death is an appropriate response to an overwhelming foreign DNA burden, whether it is perceived as an indication of infection or as mutagenic danger. Given that AIM2 is a mammalian protein, we hypothesised that responses to foreign DNA have evolved independently in different lineages. We therefore examined insect cells for cytosolic DNA-induced cell death. *Drosophila* S2 cells were killed by transfection of DNA from a range of natural sources, but the structures and sequences of DNA inducing death were quite distinct from mammalian AIM2 responses. The mode of death was rapid and lytic, and clearly distinct from apoptosis. We have defined a novel defence against invading DNA in *Drosophila.* A mode of death resembling programmed necrosis has not previously been described in insects.

Materials and Methods

Cell culture

S2 cells [10] were obtained from ATCC and propagated at 25°C in Schneider's medium supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin (medium components from Life Technologies). Bone marrow-derived macrophages (BMMs) from C57BL/6 mice were obtained by flushing cells from femurs and tibias, and culturing with macrophage growth factor CSF-1 for 7 days as described [11]. Mice were used under approval from the University of Queensland Animal Ethics Committee.

Nucleic acids

Calf thymus (CT) DNA, salmon sperm DNA and *E. coli* DNA were purchased from Sigma Aldrich and purified as described [12]. Polyinosinic:polycytidylic acid (poly(I:C)) was obtained from

Invivogen. S2 DNA was extracted using Proteinase K (ThermoFisher Scientific) according to the manufacturer's protocol and purified using phenol-chloroform extraction followed by diethyl ether extraction. Poly(dA), poly(dT) and poly(dC) single stranded DNA were synthesized using terminal transferase (TdT) (New England Biolabs). Oligo(dA), oligo(dT) and oligo(dC) primers (20mers at 0.3 μM) were incubated with TdT at 1 unit/μl and corresponding triphosphodeoxynucleotides (1mM for dATP and 4 mM for dTTP and dCTP) at 37°C overnight. Purification was done using phenol-chloroform extraction followed by ethanol precipitation. The length of the synthesized ssDNA was assessed by annealing the synthetic homopolymers to complementary 20-mer primers and visualising by standard agarose gel electrophoresis. PolydA:dT heteroduplex was obtained by annealing purified polydA and polydT in a 1:1 ratio at 37°C overnight. Double stranded status was confirmed with S1 nuclease. Synthetic random single stranded DNA was obtained using TdT elongation of oligo$(dA)_{20}$ in presence of dATP, dCTP, dGTP and with or without dTTP (2:3:3:6 molar ratio, due to preference of TdT for incorporation of individual bases). All synthesized DNA were purified with phenol-chloroform extraction. Randomly synthesized DNAs were further purified using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-30 membrane (Merck Millipore). Determination of concentration of random polymers assumed equal representation of all bases. pBlueScript (pBS) DNA was purified using an EndoFree Plasmid Maxi Kit (QIAGEN) from overnight *E. coli* culture. Methylation of pBS was done using CpG-methyltransferase *M.SssI* (New England Biolabs), followed by enzyme inactivation for 20 min at 65°C. DNA concentrations were either estimated by A260, or PicoGreen assay (Life Technologies) which specifically measures dsDNA. Cyclic dinucleotides were prepared by Zhao-Xun Liang, Nanyang Technological University, Singapore [13].

Electroporation

D. melanogaster S2 cells, hemocytes and mouse BMM were electroporated with various nucleic acids or cyclic dinucleotides in 400 μl of growth medium at 400 V, 500 μF (S2 cells and hemocytes)

or 240V, 1000 µF (BMM) using a BioRad GenePulser MX. Cells were incubated at room temperature with nucleic acid for 10 min prior to electroporation. 200,000 S2 cells or 100,000 BMMs were then plated in 96-well tissue culture plates.

Chemical transfection

For chemical transfection 250,000 S2 cells were plated per well in 100 µl Schneider's media without FCS and antibiotics and transfected with nucleic acids or cyclic dinucleotides complexed with Lipofectamine 2000 (Life Technologies). Ratios of Lipofectamine to nucleic acid of 2:1 (v/w) for nucleic acids and 1:1 (v/w) for cyclic dinucleotides. Plates containing cells and lipofectamine complexes were centrifuged at 500g for 10 min to enhance transfection, and incubated at 25°C for 18 h.

MTT assay for viability

Cleavage of tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used as an indicator of cell viability [14]. MTT is cleaved by a mitochondrial enzyme succinate dehydrogenase and produces an insoluble blue product. MTT was added to a final concentration 1 mg/ml, and cells were incubated for 1 h, or 40 min in the case of BMMs. After incubation, cells were solubilized overnight by using equal volume of MTT Solubilization Solution (10% Triton X-100 and 0.1N HCl in isopropanol). Absorbance was measured at 570 nm.

Flow cytometric analysis of cell death

For measurement of annexin V/ propidium iodide (PI) staining by flow cytometry, 200,000-300,000 S2 cells were centrifuged at 500g for 5 min, washed with 1ml of PBS, and resuspended in 100 µl of 1x Binding Buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) with 1 µl of Annexin V-Alexa Fluor 488 (Life Technologies) per sample. Samples were incubated on ice for 20min in the dark. 400 µl 1x Binding Buffer with PI (final concentration 1µg/ml) was added and

samples were analysed by flow cytometry (Accuri C6, BD Bioscience). The threshold levels for Annexin V positivity were set based on the Annexin V staining of the membrane permeable PI-positive cells, which have full exposure of phosphatidylserine to Annexin V. PI single staining was used to assess the time course of cell death. After electroporation (as described above) 200 µl of cell suspension was diluted with 200 µl of full Schneider's media with PI (final concentration 1 µg/ml). Cell suspension was analysed by flow cytometry at every 5 min after electroporation. During analysis samples were at room temperature. Pan-caspase inhibitor Z-VAD-FMK (Calbiochem) was used to test the involvement of caspases in DNA-induced cell death after electroporation. Actinomycin D (Boehringer-Mannheim) was used for inducing apoptosis in S2 cells.

SubG$_0$/G$_1$ DNA analysis [15]

10^6 cells were centrifuged at 500g for 5 min, washed with PBS, resuspended in 50 µl of PBS, and fixed with 1 ml of 100% ice-cold ethanol. Samples were stored at 4°C overnight. Cells were centrifuged at 800g for 5 min, washed with PBS with 10% FCS, centrifuged again, and resuspended in citrate buffer (38 mM trisodium citrate) with 70 µM PI and RNase A (10 µg/ml). Samples were analysed by flow cytometry (FACS Canto II, BD Biosciences).

Hemocyte extraction

For hemocyte collection, the fly line CG-GAL4 that expresses GFP in cells of the immune system (hemocyte, lymph gland and fat body) was used. The method was based on work of Lanot *et al.* [16]. Each larva was washed to remove food particles in water followed by 1x PBS and then sterilized in 70% ethanol for 2-3 min. Remains of ethanol were removed by washing in water and 1x PBS. Then the larva was placed onto a clean surface in a drop of sterile 1x PBS. Thin forceps were used to pull apart the cuticle and the larva was left in the drop for 3-5 min to collect the hemolymph. Collected hemolymph was stored on ice. The average number of larvae used in one experiment was 30-50.

Real time PCR

Expression of IFN-ß and HPRT mRNAs were determined by real time PCR as described [3], using mouse IFN-ß primers: CCACAGCCCTCTCCATCAAC, TGAAGTCCGCCCTGTAGGTG.

Statistics

To compensate for any cell number differences between experiments, MTT assay data is presented normalised to the control sample. Statistical significance was determined prior to normalisation by paired t-test, one-tailed in Figure 1, and two-tailed in Figure 2. Analysis was done using R statistical software.

Results

S2 cells die rapidly following DNA transfection

We electroporated S2 cells [10], a macrophage-like cell line derived from the late embryonic stage of *D. melanogaster*, with calf thymus DNA (CT DNA). S2 cells can be transfected with plasmids for expression of recombinant proteins, thus we expected only limited DNA-dependent death in this immortal cell line. Assessment of cell viability at 1 h post electroporation revealed that cytosolic DNA induced rapid dose-dependent cell death (Figures 1A and 1B). DNase I-treated CT DNA did not induce cell death, confirming that the effect is due to DNA, and not a contaminant in the CT DNA preparation (Figure 1C). Poly(I:C), a synthetic dsRNA, served as a control for the presence of a negatively charged polymer as a death-inducing factor. S2 cells were not sensitive to poly(I:C), even at 3 h after electroporation (Figure 1D). For comparison, similar experiments were performed

with mouse bone marrow-derived macrophages (BMM). Both dsDNA and dsRNA were toxic to mouse cells, but with differing time courses for cell death (Figure 1E). Electroporated dsDNA kills BMM via AIM2-dependent pyroptosis within 1 h [8], but with poly(I:C), cell viability was reduced only at 3 h after electroporation. Analysis of nuclear morphology confirmed that the dsRNA induced apoptosis of BMM (result not shown), explaining the slower response. Thus mouse BMM and *Drosophila* S2 cells both showed rapid death in response to DNA, but had contrasting responses to dsRNA.

As another method of nucleic acid delivery S2 cells were transfected with a cationic liposome reagent. At 18 h after transfection with CT DNA, cell viability was reduced, compared to the mock-transfected controls (Figure 1F). Poly(I:C) was used in this experiment as a control polyanion capable of forming a similar complex with transfection reagent. As for electroporation, the observed toxicity was DNA-specific, and not observed with dsRNA. Differences between time of cell death in electroporated and chemically tranfected S2 cells is presumably due to the mechanisms of DNA introduction into cells. Electroporation permeabilises the plasma membrane, which allows direct and synchronous DNA delivery into the cytosol. Using a transfection reagent requires uptake of the DNA-reagent complex and escape from the endosomal pathway, which would take longer to reach a toxic threshold of DNA.

Effects of DNA source, sequence, and strandedness on S2 cell viability

dsDNA from other sources was used to show that the observed cell death is not specific to bovine DNA. CT DNA, salmon sperm DNA, DNA isolated from S2 cells, *E. coli* genomic DNA or plasmid DNA (pBS) all induced cell death (Figure 2A). *E.coli* DNA, plasmid DNA and *Drosophila* DNA all lack the methylation of cytosine in CpG motifs seen in vertebrates, although some other insects and invertebrates exhibit CpG methylation [17]. Since *E. coli* and plasmid DNAs were the least toxic, we examined the role of CpG methylation. Plasmid DNA was treated with CpG-

methyltransferase *M.Sss*I, and the reaction was shown to be complete, by lack of digestion by methylation-sensitive restriction enzyme *Hpa*II (result not shown). Electroporation of S2 cells with equal amounts of plasmid DNA or CpG-methylated plasmid DNA revealed that CpG methylation status had no effect on the toxicity of plasmid DNA (Figure 2B).

In mammalian macrophages cell death follows recognition of cytosolic DNA by AIM2, which mediates responses only to dsDNA, and not single stranded DNA (ssDNA) [3,18]. To investigate the dependence of S2 cell death on DNA strandedness we denatured CT by boiling, transferring the DNA to ice for less than 2 min prior to electroporation into cells to minimise re-annealing. Boiled DNA had little toxic effect on BMMs, consistent with the known specificity of AIM2 responses for dsDNA [3,18] (Figure 2C). However, S2 cells were sensitive to both boiled and intact CT DNA (Figure 2D), suggesting that ssDNA was still toxic. To investigate the effect of strandedness further we used synthetic DNA. In our previous work on mouse macrophage cytosolic DNA responses, synthetic ssDNA, poly(dA), was inactive, whilst a duplex of annealed homopolymers, poly(dA):(dT) was the most toxic DNA identified [3]. Because long poly(dA) and poly(dT) homopolymers are no longer commercially available, they were generated using terminal transferase. The lengths of synthesized homopolymers were approximately 2.5kb (data not shown). Transfection of BMM with these single and double stranded homopolymers confirmed previous results of high poly(dA):(dT) toxicity [3], indicating a successful synthesis (Figure 2E). In contrast, poly(dA):(dT) had low but measurable toxicity in S2 cells (Figure 2F). There was no death of S2 cells with poly(dA) or poly(dC), and a minor effect of poly(dT) which did not reach significance (Figures 2F and G).

To investigate the effect of base composition, we generated two different mixed base DNAs with terminal transferase, one contained all nucleotides (pN) while the other lacked thymidine nucleotides (pGCA). The role of thymine was tested, because amongst the homopolymers, only poly(dT) gave any suggestion of toxicity (Figure 2F). Whilst we thought that the procedure would

generate random base ssDNA, digestion with S1 nuclease suggested the presence of significant ds regions (not shown). Nevertheless this showed that DNA of random sequence and mixed ss/ds characteristics could achieve some toxicity, but the presence of thymine bases was required for DNA toxicity (Figure 2H). Overall, the results suggest that *Drosophila* cells respond to both single-stranded and double-stranded DNA, but in a sequence-dependent manner such that homopolymers are not toxic.

DNA-induced S2 cell death is necrotic-like and not apoptotic

In order to characterise the mechanism of cell death, we stained cells with Annexin V and the membrane impermeable DNA stain, propidium iodide (PI). Annexin V binds to phosphatidylserine, and early apoptotic cells are identified as AnnexinV positive/PI negative [8]. In contrast, cells dying via necrosis or pyroptosis rapidly lose membrane integrity and stain with PI and Annexin V simultaneously. S2 cells were stained with Annexin V and PI 30 min after electroporation. DNA-treated cells rapidly lost their membrane integrity, and no significant population of cells displayed an apoptotic Annexin V-positive/PI-negative profile (Figure 3A). In apoptosis, loss of membrane integrity is not seen within 30 min of the stimulus [8]. As a control, staining was conducted on S2 cells that were treated with actinomycin D for 6 h, revealing the expected population of annexin V-positive/PI-negative apoptotic cells (Figure 3A). In summary, DNA induced cell death proceeds via a mechanism involving rapid loss of membrane integrity, lacking an Annexin V-positive/PI-negative population and hence is distinct from apoptosis.

We examined the kinetics of loss of membrane integrity. S2 cells were electroporated with and without CT DNA then incubated at room temperature with PI and analysed by flow cytometry every 5 min to differentiate live and dead cells (Figure 3B). After 5 min there was a modest increase in the percentage of dead cells in electroporated samples compared to untreated (un-electroporated cells) and this was relatively constant over the following 30 min of incubation. Electroporation with

DNA resulted in an increase in PI positive cells that first became apparent from 20 min post electroporation. The incubation temperature of cells was 4˚C lower than in Figure 3A, and this is likely to affect the rate of death. Electroporation with the dsRNA analogue poly(I:C) (Figure 3C) did not result in a progressive increase in dead cells, confirming the response was specific to dsDNA (Figure 3B).

To confirm the non-apoptotic nature of this cell death, we analysed DNA content of cells by flow cytometry. Cells undergoing later stages of apoptosis can be detected as nuclei with sub-G_0/G_1 DNA due to apoptotic inter-nucleosomal DNA cleavage, and loss of small fragments of DNA during fixation [15]. S2 cells were electroporated with CT DNA or poly(I:C), or treated with actinomycin D as a positive control, and incubated for 18 h prior to processing. Whilst nuclei with sub-G_0/G_1 DNA are clearly present in actinomycin D-treated samples (Figure 3D), DNA-induced cell death did not produce the sub-G_0/G_1 cell fraction that is characteristic of apoptosis. Electroporation alone induced a degree of apoptosis as evident by the appearance of sub-G_0/G_1 DNA, however the inclusion of DNA in the electroporation apparently reduced such apoptosis, presumably as the cells had already rapidly died by another route. These results confirmed that cytosolic DNA induces a type of cell death that is phenotypically distinct from apoptosis.

Activation of caspases initiates apoptosis in both flies and mammals. Z-VAD-FMK, a pan-caspase inhibitor, was employed to investigate caspase-dependence of DNA-dependent cell death. S2 cells were pretreated with Z-VAD-FMK or DMSO as a control for 1 h before subsequent treatments and flow cytometric analysis with PI to measure cell death. Z-VAD-FMK prevented death induced by actinomycin D (Figure 4A), which proceeds by an apoptotic route (Figure 3B). In contrast, Z-VAD-FMK did not prevent DNA-induced cell death measured at 30 min post electroporation (Figure 4B), suggesting a lack of requirements for typical apoptotic caspases. Overall, DNA-induced death exhibits no characteristics of apoptosis, and the rapid loss of membrane integrity demonstrates a

necrotic style of death previously undescribed in insects.

Known pathways do not explain S2 cell death

Since pathways can be reused for new purposes in evolution, we considered whether a STING-dependent pathway could contribute to *Drosophila* DNA-induced death. Mammalian STING recognises bacterial second messengers cyclic di-GMP and cyclic di-AMP in addition to the ligand generated by cGAS following DNA recognition. An uncharacterised STING homologue is present in the fly genome. Cyclic di-GMP and cyclic di-AMP [13] were introduced into S2 cells using electroporation and chemical transfection but had no toxic effect on S2 cells (Figures 5A and B). Under similar conditions cyclic di-GMP transfected into BMM strongly induced IFN-β mRNA (Figure 5C). We conclude that DNA-induced cyclic dinucleotides and a STING-dependent pathway are unlikely to play a role in *Drosophila* DNA-induced cell death.

Drosophila primary hemocytes display DNA-dependent cell death

Responses to foreign DNA have been predominantly characterised in mammalian macrophages. The phagocytic innate immune cells in *D. melanogaster* are hemocytes. We isolated hemocytes and tested their responses to transfected DNA. Hemocytes were electroporated with either CT DNA, DNase I-treated, or mock DNAse I-treated CT DNA. Flow cytometric analysis revealed that hemocytes died in response to transfected DNA (Figure 6), and that DNAse I treatment abolished this sensitivity. Electroporation with DNA that had been mock DNAse I-treated retained toxicity, confirming that components of the digest did not prevent DNA toxicity. This establishes that the DNA-induced death observed in S2 cells is relevant to primary *Drosophila* hemocytes.

Discussion

Recognition of cytosolic foreign DNA is of demonstrated importance in a number of bacterial and viral infections of mouse cells [19,20], and even prokaryotes have restriction-modification and CRISPR systems for defence against foreign DNA. Thus responses to invading DNA might be anticipated to be widespread in nature. The insect cell death in response to electroporated DNA observed here was of similar sensitivity and rapidity to AIM2-mediated death of mouse macrophages [3]. AIM2 responses mediate protection against both viruses and cytosolic bacteria [20], and our work suggests insect cells will have a similar DNA-mediated defence. A DNA virus naturally infecting *Drosophila* species has been recently identified [21], but recent work showing that positive strand RNA viruses generate viral cDNA using endogenous reverse transcriptases [22], extends the relevance of DNA recognition pathways.

The insect cell recognition of DNA was demonstrably different from a mammalian AIM2 response. AIM2 recognises only dsDNA, and responses are lost with freshly boiled DNA [18]. In contrast denatured DNA retained activity on *Drosophila* cells. In addition, the strongest ligand we have found for murine AIM2 is dsDNA obtained by annealing of two synthetic homopolymers to give poly(dA):(dT) [3]. This double stranded homopolymer is known to have an altered helical pitch compared to normal B-form DNA [23]. This may be favourable for AIM2 recognition, but was only a weak ligand for *Drosophila* cell death. No synthetic DNA approached the toxicity of natural DNAs, suggesting that particular structures or sequences not frequent in a random sequence may be involved. The omission of thymine from randomly synthesised DNA suggested that this base was essential for the *Drosophila* responses.

For many years apoptosis was the only described route of programmed cell death. Apoptosis is caspase-dependent cell death characterized by cell shrinkage and blebbing, nuclear condensation and fragmentation, with nuclear DNA being cleaved in internucleosomal regions [24]. An early

marker of apoptosis is the exposure of phosphatidylserine on the outer surface of the cell membrane, which acts as an "eat-me" signal for phagocytes. If not phagocytosed, apoptotic cells eventually undergo secondary necrosis and lose membrane integrity. It is now apparent that apoptosis is not the only "purposeful" way for a cell to die, with the description of programmed necrosis (necroptosis), and caspase 1-dependent pyroptosis [25,26]. Unlike apoptosis, necrosis and pyroptosis both involve rapid loss of membrane integrity, leading to an inflammatory response. Here we found that apoptotic features were absent in DNA-dependent *Drosophila* cell death, and the cells rapidly lost membrane integrity. Apoptosis is well characterized in both mammals and flies, whereas necroptosis and pyroptosis are not described in insects. Direct orthologues of genes encoding proteins involved in necroptosis (RIPK1, RIPK3, MLKL) or pyroptosis (Caspase-1) are not found in the *Drosophila* genome. (*Drosophila* initiator caspases are more closely related to mammalian caspases-8, and -9 [27]). Thus the mechanism through which DNA induces rapid necrotic type death remains to be established. The use of a pan-caspase inhibitor failed to prevent death, suggesting lack of caspase involvement. Experimental infection of *Drosophila* with a lepidopteran baculovirus suggests apoptosis is a host defence [28,29], but viral studies have not yet identified rapid lytic insect cell death. Cell death is an important defence against intracellular pathogens, and simultaneous induction of multiple cell death pathways is likely as a host response to pathogen evasion strategies.

The DNA-induced death pathway described here for *Drosophila* may be more widely relevant; we have recently found DNA-induced death in chicken macrophages is similarly sensitive to denatured DNA and independent of AIM2 (manuscript in preparation). Elucidation of the molecular details of this pathway cannot readily exploit the use of *Drosophila* mutant libraries, since a high throughput screen for response to transfected DNA is not feasible. However, direct affinity purification of cytosolic DNA binding proteins can be pursued [3], and candidate genes assessed with knockouts or knockdown.

Despite a lack of characterised DNA receptor, there is some evidence suggesting other responses to non-chromosomal DNA in *Drosophila*. DNase II is a lysosomal DNase involved in the degradation of phagocytosed DNA [30]. DNA within apoptotic cells is cut into nucleosomal units by caspase-activated DNase (CAD), and further DNase II-dependent degradation occurs following uptake into a macrophage phagolysosome [30,31]. A mutation in the *Drosophila* DNAse II orthologue that leads to diminished enzyme activity, results in accumulation of DNA in apoptotic cells in the ovary, increased susceptibility to bacterial infections, reduced number of hemocytes, and elevated levels of the antimicrobial peptides (AMPs) diptericin and attacin A [32,33]. The induction of AMPs was further increased in DNase II mutant flies without functional *Drosophila* caspase-activated DNase (dCAD) [32]. This presents an interesting parallel to the DNase II knockout mouse, that has elevated expression of type I IFNs, causing embryonic lethality [34]. The mouse phenotype is independent of toll-like receptors that might respond to DNA, but dependent on STING, implicating the involvement of the cGAS pathway of recognition of cytosolic DNA leading to IFN-β production [2,35,36]. Thus it is likely that accumulated DNA in the phagolysosome of DNase II-deficient mouse or *Drosophila* cells escapes to the cytosol for recognition. A role for STING has not been investigated in the DNase II mutant fly, but we showed it is unlikely to play a role in DNA-induced cell death. However, given the likely escape of undigested DNA into the cytosol of DNase II mutant cells, the cell death pathway we have described is consistent with the documented low level of hemocytes in the DNase II mutant flies [33].

Apart from response to infection, DNA-induced death could equally well have evolved to protect the genome from invading (retro)transposons. Although newly acquired DNA can drive evolution, the too rapid accumulation of DNA insertions could lead to loss of viability. DNA insertions into the germline have obvious consequences for organism viability and evolution, but insertions in

somatic cells can drive tumourigenesis or lead to loss of function during the life of the animal. Consequently organisms should limit integration of exogenous DNA, or the re-integration of endogenous retroelements, into their genome. The nuclease Trex1 (DNase III) that is involved in the degradation of cDNA of invading retroviruses and endogenous retroelements [37,38] may provide one level of defence. However, if the burden of unintegrated DNA remains high, cell death may protect the organism from replicating retrotransposons, or may protect unicellular organisms at the population level. Elucidation of the DNA recognition pathway as well as the specific DNA sequences that drive insect cell death will aid the understanding of the evolution of these responses.

Acknowledgements

This project was funded by Australian Research Council (ARC) Grant DP120103332. KJS was supported by ARC and NHMRC Fellowships FT0991576 and 1059729. Cyclic dinucleotides were kindly provided by Zhao-Xun Liang, Nanyang Technological University, Singapore. The authors have no financial conflicts of interest relating to the results of this work.

References

1 Gasiunas G, Sinkunas T, Siksnys V: Molecular mechanisms of CRISPR-mediated microbial immunity. Cell Mol Life Sci 2014;71:449-465.
2 Sun L, Wu J, Du F, Chen X, Chen ZJ: Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 2013;339:786-791.
3 Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ: HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 2009;323:1057-1060.
4 Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA: AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 2009;458:514-518.
5 Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES: AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009;458:509-513.
6 Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL, Superti-Furga G: An orthogonal proteomic-genomic screen

identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol 2009;10:266-272.
7 Miao EA, Rajan JV, Aderem A: Caspase-1-induced pyroptotic cell death. Immunol Rev 2011;243:206-214.
8 Sagulenko V, Thygesen SJ, Sester DP, Idris A, Cridland JA, Vajjhala PR, Roberts TL, Schroder K, Vince JE, Hill JM, Silke J, Stacey KJ: AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. Cell Death Differ 2013;20:1149-1160.
9 Cridland JA, Curley EZ, Wykes MN, Schroder K, Sweet MJ, Roberts TL, Ragan MA, Kassahn KS, Stacey KJ: The mammalian PYHIN gene family: phylogeny, evolution and expression. BMC Evol Biol 2012;12:140.
10 Schneider I: Cell lines derived from late embryonic stages of Drosophila melanogaster. J Embryol Exp Morphol 1972;27:353-365.
11 Costelloe EO, Stacey KJ, Antalis TM, Hume DA: Regulation of the plasminogen activator inhibitor-2 (PAI-2) gene in murine macrophages. Demonstration of a novel pattern of responsiveness to bacterial endotoxin. J Leukoc Biol 1999;66:172-182.
12 Stacey KJ, Young GR, Clark F, Sester DP, Roberts TL, Naik S, Sweet MJ, Hume DA: The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. J Immunol 2003;170:3614-3620.
13 Rao F, Pasunooti S, Ng Y, Zhuo W, Lim L, Liu AW, Liang ZX: Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. Anal Biochem 2009;389:138-142.
14 Denizot F, Lang R: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271-277.
15 Riccardi C, Nicoletti I: Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 2006;1:1458-1461.
16 Lanot R, Zachary D, Holder F, Meister M: Postembryonic hematopoiesis in Drosophila. Dev Biol 2001;230:243-257.
17 Raddatz G, Guzzardo PM, Olova N, Fantappie MR, Rampp M, Schaefer M, Reik W, Hannon GJ, Lyko F: Dnmt2-dependent methylomes lack defined DNA methylation patterns. Proc Natl Acad Sci U S A 2013;110:8627-8631.
18 Stacey KJ, Ross IL, Hume DA: Electroporation and DNA-dependent cell death in murine macrophages. Immunol Cell Biol 1993;71 (Pt 2):75-85.
19 Gao D, Wu J, Wu YT, Du F, Aroh C, Yan N, Sun L, Chen ZJ: Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. Science 2013;341:903-906.
20 Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA: The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 2010;11:395-402.
21 Unckless RL: A DNA virus of Drosophila. PLoS One 2011;6:e26564.
22 Goic B, Vodovar N, Mondotte JA, Monot C, Frangeul L, Blanc H, Gausson V, Vera-Otarola J, Cristofari G, Saleh MC: RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat Immunol 2013;14:396-403.
23 Nelson HC, Finch JT, Luisi BF, Klug A: The structure of an oligo(dA).oligo(dT) tract and its biological implications. Nature 1987;330:221-226.
24 Strasser A, Cory S, Adams JM: Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. Embo J 2011;30:3667-3683.
25 Aachoui Y, Sagulenko V, Miao EA, Stacey KJ: Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. Curr Opin Microbiol 2013;16:319-326.
26 Vanlangenakker N, Vanden Berghe T, Vandenabeele P: Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 2012;19:75-86.
27 Denton D, Aung-Htut MT, Kumar S: Developmentally programmed cell death in Drosophila. Biochim Biophys Acta 2013;1833:3499-3506.

28 Lamiable O, Imler JL: Induced antiviral innate immunity in Drosophila. Curr Opin Microbiol 2014;20C:62-68.
29 Liu B, Behura SK, Clem RJ, Schneemann A, Becnel J, Severson DW, Zhou L: P53-mediated rapid induction of apoptosis conveys resistance to viral infection in Drosophila melanogaster. PLoS Pathog 2013;9:e1003137.
30 Evans CJ, Aguilera RJ: DNase II: genes, enzymes and function. Gene 2003;322:1-15.
31 Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H: Degradation of chromosomal DNA during apoptosis. Cell Death Differ 2003;10:108-116.
32 Mukae N, Yokoyama H, Yokokura T, Sakoyama Y, Nagata S: Activation of the innate immunity in Drosophila by endogenous chromosomal DNA that escaped apoptotic degradation. Genes Dev 2002;16:2662-2671.
33 Seong CS, Varela-Ramirez A, Aguilera RJ: DNase II deficiency impairs innate immune function in Drosophila. Cell Immunol 2006;240:5-13.
34 Yoshida H, Okabe Y, Kawane K, Fukuyama H, Nagata S: Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. Nat Immunol 2005;6:49-56.
35 Ahn J, Gutman D, Saijo S, Barber GN: STING manifests self DNA-dependent inflammatory disease. Proc Natl Acad Sci U S A 2012;109:19386-19391.
36 Okabe Y, Kawane K, Akira S, Taniguchi T, Nagata S: Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. J Exp Med 2005;202:1333-1339.
37 Stetson DB, Ko JS, Heidmann T, Medzhitov R: Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell 2008;134:587-598.
38 Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J: The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nat Immunol 2010;11:1005-1013.

Figure Legends

Figure 1. Transfected DNA rapidly kills *Drosophila* S2 cells. Viability of S2 cells was measured by MTT cleavage at 1 h post electroporation, unless otherwise stated. **A.** DNA-dependent cell death of S2 cells after electroporation with 10 μg CT DNA. Bars represent data from 40 experiments (mean ± SEM) normalised to the no DNA sample for each experiment. ****$p<10^{-15}$. **B.** Dose-dependent response of S2 cell after transfection with indicated amounts of CT DNA. A representative of 2 experiments is shown, with error bars showing the range of results for duplicate electroporations. **C.** DNA-specific response of S2 cells. Cells were electroporated with 10 μg CT DNA that was untreated or treated with DNase I. Bars represent data from 3 experiments (mean ± SEM), normalised to the no DNA sample for each experiment. **D.** S2 cells are not killed by synthetic dsRNA poly(I:C). S2 cells were electroporated with 10 μg CT DNA or poly(I:C), and cleavage of MTT measured after 1 and 3 h. Bars represent data from 3 experiments (mean ± SEM), normalised to the no DNA samples. **E.** Mouse BMM responses to DNA and dsRNA transfection. BMMs were electroporated as per panel D. Data is from a representative experiment and error bars show the range of duplicate electroporations. **F.** DNA-dependent cytotoxic effect in S2 cells after transfection with Lipofectamine 2000. Cells in 100 μl were transfected with 2 μg of CT DNA or poly(I:C) with 4 μl of transfection reagent, or 4 μl of transfection reagent alone (mock). Cleavage of MTT was measured at 18 h after transfection. Data is from 5 experiments (mean ± SEM), normalised to the mock sample (**p= 0.013).

Figure 2. Response of S2 cells and BMMs to different types of DNA. A. Cytotoxic effect of DNA from different sources on S2 cells. 10 μg of CT DNA, or DNA from salmon sperm, S2 cells, *E.coli* and pBlueScript plasmid (pBS) were used in electroporations. Data is from two experiments (mean ± range). **B.** Cytotoxic effect of DNA on S2 cells is not dependent on methylation. Cells were electroporated with 10 μg of CT DNA, or pBS DNA, either methylated or unmethylated on CpG sequences. Each dot represents a separate electroporated sample with different symbols

denoting 2 different experiments. **C and D.** Response of BMM (**C**) and S2 cells (**D**) to boiled DNA. 10 μg CT DNA was left intact or boiled for 10 min, transferred to ice and immediately used for electroporation. Bars represent data from 3 (**C**) and 5 (**D**) experiments (mean ± SEM), normalised to the no DNA sample (*p=0.029). **E.** Cytotoxic effect of synthetic ss- and dsDNA in BMM. 5 μg of CT DNA or synthetic DNA (poly(dA), poly(dT) or poly(dA:dT)) or 1 μg of poly(dA:dT) was electroporated. **F.** Cytotoxic effect of synthetic DNAs in S2 cells, as per panel E. Each dot represents a separate electroporated sample, with the different symbols denoting the 3 different experiments n=3, ## p=0.054. **G.** Absence of cytotoxic effect of poly(dC) on S2 cells. 5 μg of CT DNA or synthetic DNA was used for electroporation. Each dot represents a separate electroporated sample, with the different symbols denoting the different experiments. **H.** Toxic effect of synthetic mixed base DNA with or without thymidine (poly(dN) and poly(dGCA)) on S2 cells. 10μg of DNA was used for electroporation. Data is from 5 experiments (CT DNA and poly(dN), mean ± SEM **p=0.0085), or two experiments (poly(dGCA), mean ± range).

Figure 3. DNA induces cell death in S2 cells through a non-apoptotic mechanism. A. Rapid loss of membrane integrity in response to DNA transfection. S2 cells were either untreated, electroporated with no DNA (ZAP) or electroporated with 10 μg of CT DNA (DNA) and stained after 30 min. As a positive control for apoptosis, S2 cells were incubated in 1 μM actinomycin D for 6 h (Act D). Annexin V/PI stained cells were analysed by flow cytometry. Apoptotic cells are defined as annexin V^+ PI^-, necrotic (dead) as PI^+ and live as double negative. Data are representative of at least 3 experiments. **B and C.** Time course of rapid DNA-induced cell death in S2 cells. Cells were electroporated with 10 ug of CT DNA (**B**) or poly(I:C) (**C**), and compared to cells electroporated with no addition (ZAP alone), for permeability to PI during incubation at room temperature. After electroporation, cells were stained with PI and analysed by flow cytometry. Data from 3 experiments are shown (mean ± SEM), with results for PI-positive cells in an untreated sample subtracted. Data represents the increase in PI positive cells relative to untreated cells. **D.**

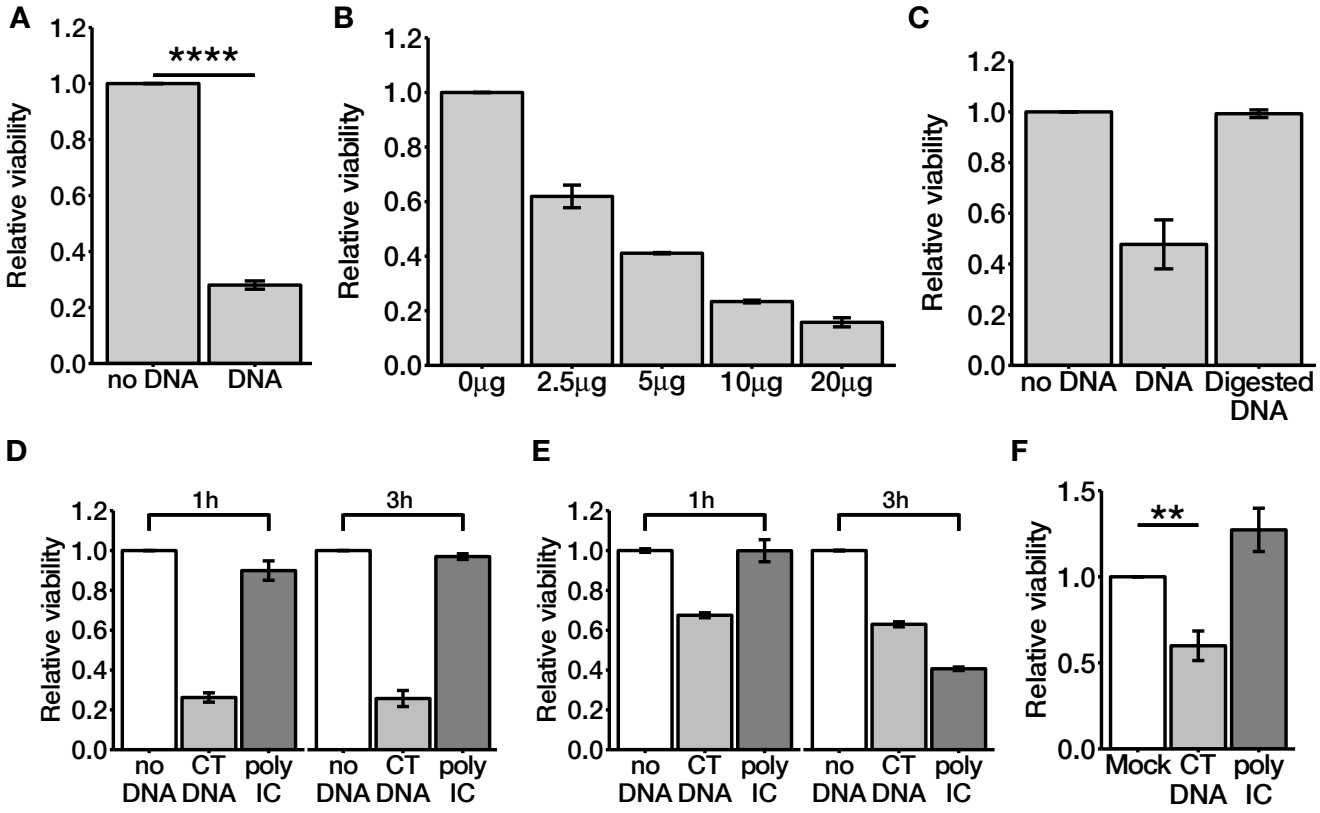
Analysis of DNA content is consistent with non-apoptotic death. S2 cells were electroporated with 10 μg CT DNA or poly(I:C) and then incubated for 18 h at 25°C. As a control for apoptosis, S2 cells were treated with 1 μM actinomycin D for 18 h. Plots show DNA content of cells based on PI staining intensity measured by flow cytometry, with the percentage of particles with a sub-G_0/G_1 level of DNA indicated. Data represent one of 3 independent experiments.

Figure 4. DNA-induced rapid cell death is not prevented by caspase inhibition. A. Actinomycin D-induced apoptosis is prevented by Z-VAD-FMK. S2 cells were treated with or without actinomycin D with either 50μM Z-VAD-FMK or DMSO vehicle control for 6 h and analysed for loss of membrane integrity by PI staining and flow cytometry. **B.** DNA-induced cell death in S2 cells is not affected by Z-VAD-FMK. S2 cells were pretreated with 50μM Z-VAD-FMK or DMSO vehicle control for 1 h prior to electroporation with 10 μg CT DNA. After 30 min they were stained with PI and analysed by flow cytometry. Data are representative of 2 experiments.

Figure 5. Cyclic dinucleotides have no toxic effect on S2 cells, and are not likely to be second messengers in the cell death response to DNA. A. Electroporation of S2 cells with CT DNA and cyclic dinucleotides. Cells were electroporated with 10 μg of CT DNA or 20 μg of cyclic di-GMP or cyclic di-AMP and MTT cleavage was measured at 1 h after treatment. Results show the mean and range of duplicate electroporations. **B.** Transfection of S2 cells with CT DNA and cyclic dinucleotides using Lipofectamine 2000. 2 μg of CT DNA and 4 μg of cyclic dinucleotides were transfected into S2 cells complexed with 4 μl of Lipofectamine 2000, and MTT cleavage was measured at 18 h post transfection. Bars show data from 4 experiments (mean ± SEM). **C.** Induction of IFN-β mRNA in BMM either untreated (no zap), or 2 hours after electroporation with either no addition, CT DNA or various amounts of cyclic di-GMP. Real time PCR results shown are mean and range of duplicate assays of IFN-β mRNA relative to HPRT.

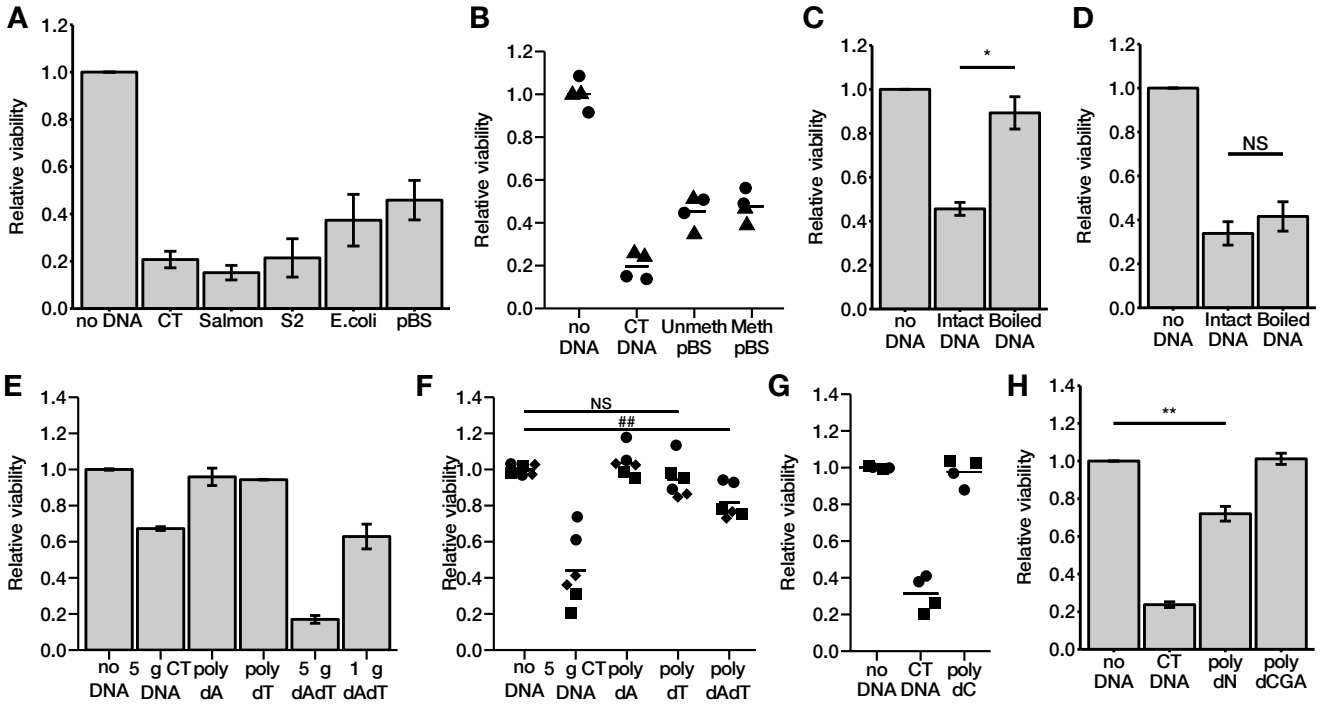
Figure 6. Primary hemocytes are sensitive to cytosolic DNA-induced death. Primary hemocytes were untreated or electroporated with no additions (ZAP alone), with 10 μg of CT DNA, CT DNA digested with DNase I, or CT DNA mixed with digestion buffer and no enzyme (mock digest). After 30 min they were stained with PI and analysed by flow cytometry. Data are representative of 2 (DNase-treated sample) or 5 (CT DNA) experiments.

Figure 1



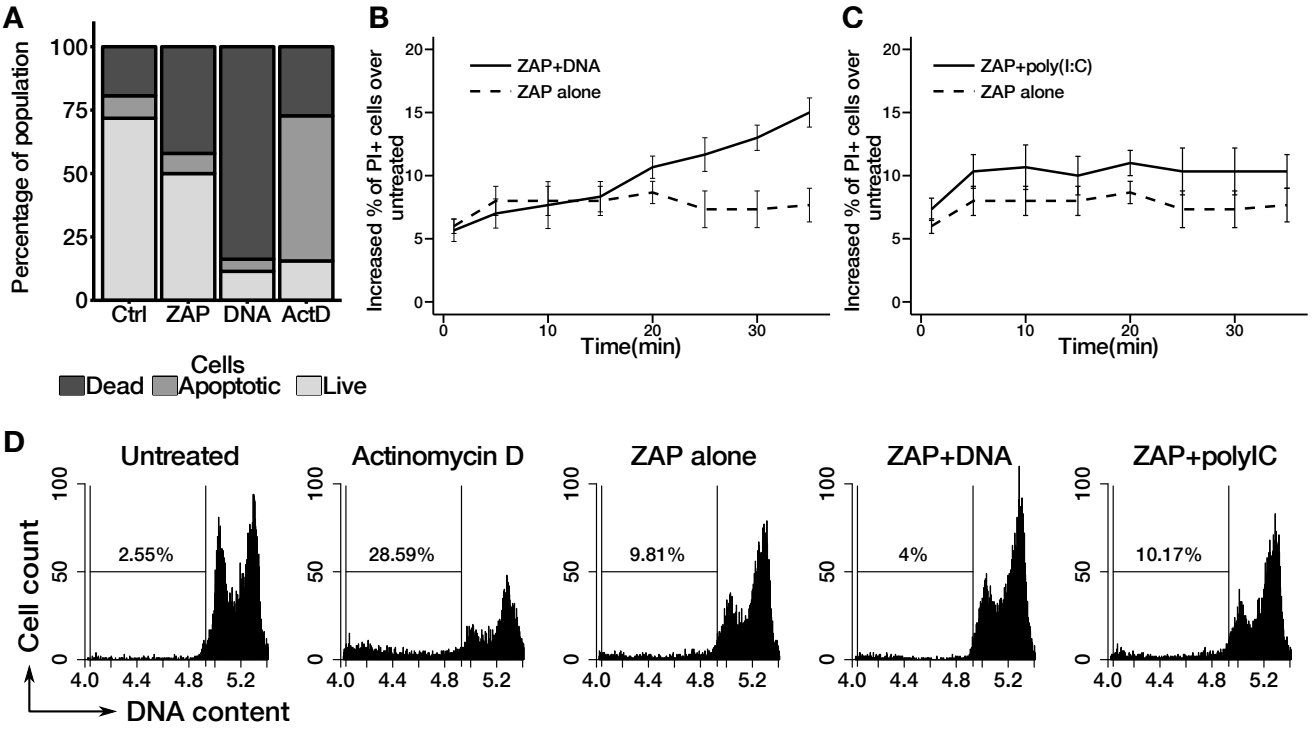
Vitak et al., 2014

Figure 2



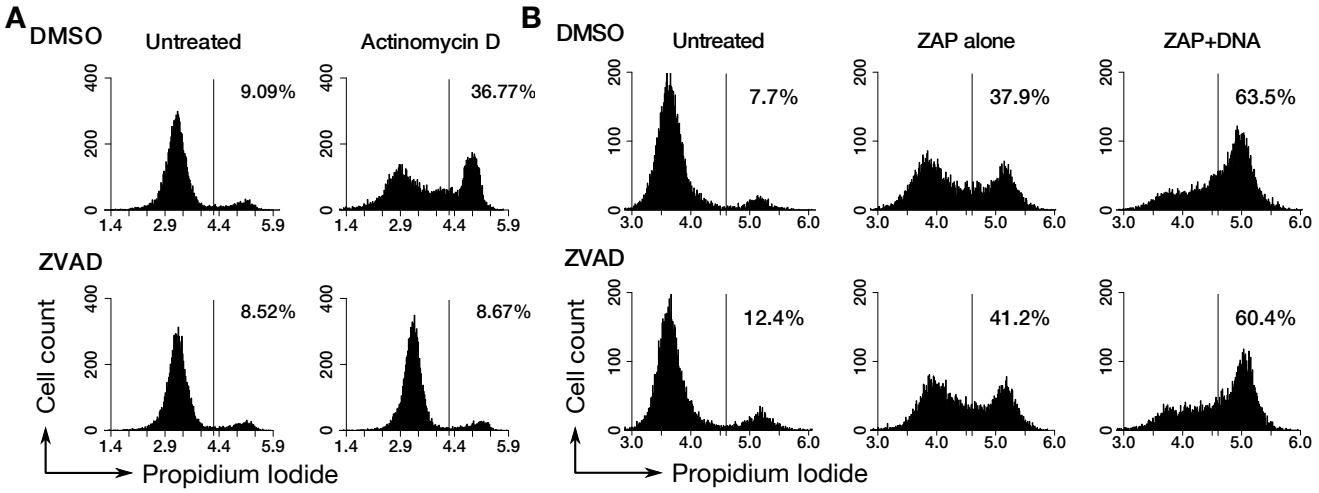
Vitak et al., 2014

Figure 3



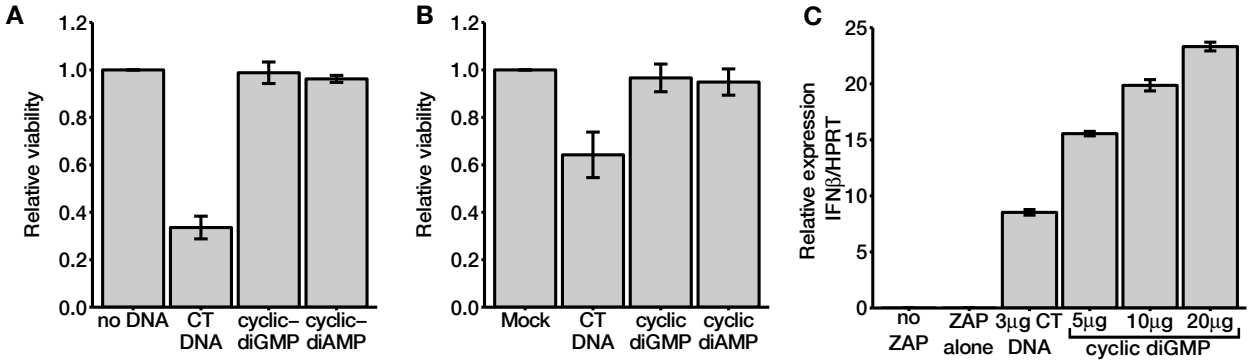
Vitak et al., 2014

Figure 4



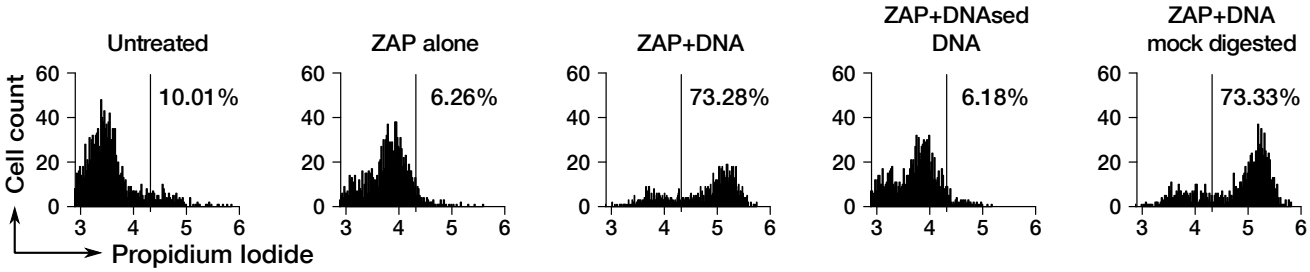
Vitak et al., 2014

Figure 5



Vitak et al., 2014

Figure 6



Vitak et al., 2014