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(54) **Title**: DNA SENSORS

(57) **Abstract**: The present disclosure relates to a DNA sensor, polynucleotide sequences rich in the motif GAGA which is detected by said DNA sensor and which stimulate immune responses, particularly innate immune responses, a protein that inhibits said DNA sensor, methods of preparing said DNA sensor, the polynucleotide and/or protein in particular by recombinant methods, compositions, such as pharmaceutical compositions comprising any one of the same, methods of eliminating or dampening the effect of said polynucleotide sequences and use of one or more of the above in treatment, for example employing a protein such as C16. In one aspect there is also provided a method of modulating the immunogenicity of a DNA virus by increasing or decreasing the number of repeats of a particular sequence and/or by the removal or insertion of virus genes encoding proteins that inhibit sensing of this sequence. The genetically engineered virus obtained from the latter also forms an aspect of the invention.

DNA sensors

The present disclosure relates to a DNA sensor, polynucleotide sequences detected by said DNA sensor which stimulate immune responses, particularly innate immune responses, a protein that inhibits said DNA sensor, methods of preparing said DNA sensor, the polynucleotide and/or protein in particular by recombinant methods, compositions, such as pharmaceutical compositions comprising any one of the same, methods of eliminating or dampening the effect of said polynucleotide sequences and use of one or more of the above in treatment, for example employing a protein such as C16. In one aspect there is also provided a method of modulating the immunogenicity of a DNA virus by increasing or decreasing the number of repeats of a particular sequence and/or by the removal or insertion of virus genes encoding proteins that inhibit sensing of this sequence. The genetically engineered virus obtained from the latter also forms an aspect of the invention.

Background

The mammalian immune system can be considered to have two arms, the innate and the adaptive systems. The innate immune system is important for the direct sensing of danger, such as components of bacterial cell walls or viral nucleic acid whereas the adaptive immune system induces antigen-specific responses to particular microbes following their detection by the innate system.

The detection of invading microbes by the innate immune system occurs via a series of pattern recognition receptors (PRRs) which bind directly to pathogen associated molecular patterns (PAMPs) (Medzhitov, 2007). PAMPs are specific molecular motifs found in microbes. Bacterial lipopolysaccharide (LPS), for example, is a component of gram negative bacterial cell walls and is detected by a PRR called Toll-like-receptor 4 (TLR-4). After engagement of TLR4, downstream adaptor molecules link the sensing of LPS to upregulation of pro-inflammatory genes within the detecting cell. This inflammatory response attracts cells of the immune system, such as neutrophils and macrophages which specialise in dealing with the particular insult (Takeda and Akira, 2007).

The detection of the nucleic acids which compose viral genomes, or are produced during virus replication, is crucial for the immediate innate immune responses to these pathogens.

What-is-more these intrinsic mechanisms have been employed in some instances for therapeutic effect, for example certain short oligonucleotides have been used as adjuvants in vaccines to boost the immune response of the recipient to active components of the vaccine. Those containing the motif CpG are known, for example from WO2006/091915 which are thought to be Toll Like Receptor 9 (TLR-9) agonists.

Viral RNA sensing mechanisms have been characterised. Extracellular RNA is detected by Toll-like-receptors 3 & 7 which then activate downstream signalling pathways to induce the expression of various cytokines, interferons, and chemokines (Alexopoulou et al., 2001). Intracellular RNA is recognised by RIG-I and MDA-5. RIG-I recognises 5'triphosphate RNA (a feature of many virus genomes such as influenza) whereas MDA-5 recognises long double stranded RNA (Kato et al., 2006). After recognition of the cognate ligand by either RIG-I or MDA-5 the adaptor molecule IPS-1 links these receptors to activation of transcription factors IRF3 and NF-κB. These two factors bind to consensus sequences within inflammatory genes and induce their transcription (Kawai et al., 2005).

The molecular mechanisms of intracellular DNA detection has been under intense study in recent years since the discovery that intracellular DNA can activate IRF3 leading to the upregulation of inflammatory genes (Ishii et al., 2006; Stetson and Medzhitov, 2006). The receptors responsible for detecting this DNA, however, remained unidentified until recently when a series of DNA-sensors were discovered:

- DAI was found to bind to DNA and to be responsible for activation of the innate immune response to poly (dA:dT) DNA in certain cell types, however mice lacking DAI showed no reduction in the activation of an innate immune response by various viruses indicating additional sensors were likely to exist (Takaoka et al., 2007; Wang et al., 2008).
- AIM2 is a DNA sensor found predominantly in macrophages which detects DNA and activates inflammasome-dependent IL-1β and IL-18 secretion. AIM2 works independently of the IRF3-dependent interferon response (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).
- RNA-polymerase III recognises AT-rich DNA in the cytoplasm and transcribes it into 5'triphosphate RNA, which acts as a ligand for the RNA sensing machinery and leads to IRF3 and NF-κB activation (Ablasser et al., 2009; Chiu et al., 2009).
- LRRFIP1 is predominantly found in macrophages and, upon DNA binding, activates the transcription of interferon via a beta-catenin-dependent pathway (Yang et al. 2010)
- IFI16 is a molecule not shown to demonstrate any specificity for a particular type of DNA and has been studied mostly in macrophages to activate IRF3 and lead to an antiviral pro-inflammatory response (Unterholzner et al. 2010).

The present inventors have identified a novel DNA sensor, DNA-PK, which is shown to activate IRF3 after detection of cytoplasmic DNA.

Proteins Ku70 and Ku80/86 are known in the art to play a role in DNA repair and to be important for the adaptive immune response, for instance for the development of antibody diversity, which requires DNA joining. Together Ku70 and Ku80/86 proteins are referred to as the Ku complex. The Ku complex binds to the catalytic subunit DNA-PKcs (a DNA dependent protein kinase) to form a heterotrimer.

The present inventors have established that the absence of any of these subunits dramatically reduces upregulation of cytokines and chemokines in response to DNA transfection and infection by a cytoplamic DNA virus. This novel activity seems to be unrelated to function of DNA repair.

A mutant mouse wherein the kinase activity of DNA-PK was removed by deleting the last few amino acids from DNA-PKcs, retained DNA sensing and innate immune activity. This may indicate that innate immune response generated by DNA-PKs works via a different mechanism to its previously known functions. In fact these functions appear to work independently of each other.

It is known that the Ku complex portion of DNA-PK binds to DNA in a sequence independent manner, but has a preference for a sequence termed NRE1. The NRE1 sequence contains the sequence 5' GAGAAAGAGAA 3' SEQ ID NO: 1 (hereafter termed 'GAGA').

This sequence occurs naturally in some organisms, in particular viruses. The number of copies of this sequence varies, for example this sequence is found in high copy number in some

poxvirus genomes, notably vaccinia virus (VACV) strain modified virus Ankara (MVA), but in variola virus (the cause of the disease smallpox) there is only a single copy of this sequence. MVA is very immunogenic despite not replicating in most mammalian cells including most human cells and it had been hypothesized previously that this high immunogenicity was due to the loss of genes encoding proteins that inhibit the innate immune response to infection. The present inventors hypothesise that this high immunogenicity may alternatively or additionally be related to the high number of copies of this sequence in this virus.

NRE1 was found by the present inventors to have a higher potency for activating the immune system compared to a control DNA sequence and this clearly has application in therapy. What is more it gives access to a method of modulating the immunogenicity of DNA viruses or other DNA containing agents such as bacteria or naked DNA such as plasmids or a DNA fragment, by deleting or inserting NRE1 sequences.

Finally, the VACV strain Western Reserve (WR) C16 protein (hereinafter referred to as C16) has been characterized and its effects on virus replication and virulence have been reported (Fahy et al., J Gen Virol 2008). The present inventors have now established the mechanism by which C16 acts to promote virus virulence. It is shown that it interacts with the Ku complex during VACV infection, or following its introduction into uninfected cells, and that C16 blocks innate immune sensing of dsDNA. Thus the C16 protein appears to act on the Ku complex to downregulate innate immune responses during viral infection.

Thus the present inventors have identified a DNA sensor which preferentially binds a polynucleotide sequence which in turn stimulates immune responses, the polynucleotide sequence is characterised by the motif 5' GAGAAAGAGAA 3'. There is also disclosed an inhibitor of the DNA sensor.

Summary of the Disclosure

There is provided an isolated polynucleotide sequence comprising a region rich in the motif GAGA for example comprising the motif $([X]_{0-50}GAGAAAGAGAA[Y]_{0-50})n$ [SEQ ID NO: 2] wherein n is an integer 0 to 150, X or each X is independently selected from a base and Y or each Y is independently selected from a base, for example wherein said sequence is suitable for generating an innate immune response *in vivo*.

Brief Description of the Figures

Figure 1 Shows that the GAGA sequence increases the potency of the innate immune response of cells compared with a control DNA sequence.

Double stranded DNA oligonucleotides (100 bp) containing control sequence (ISD), 50 bp control sequence and 50 bp of the GAGA sequence repeated (50% GAGA) and 100 bp of GAGA (100% GAGA) were transfected into cells and the upregulation of the CXCL-10 gene transcription was measured using real time reverse-transcription-PCR.

Figure 2 shows that the DNA target sequence for Ku (NRE1) is immunostimulatory.

It has been previously reported that Ku binds particularly well to the NRE1 sequence in the murine mammary tumour virus. Interestingly, a sequence within the NRE1 sequence is also found in some poxvirus genomes as well as the genomes of other DNA viruses. It was proposed that since Ku binds the NRE1 DNA sequence preferentially and that Ku activates the immune system in response to

DNA, the NRE1 sequence might be particularly immunostimulatory. Stimulation of MEFs (mouse embryonic fibroblasts) with either a control (ISD) DNA or NRE1 DNA lead to the observation that NRE1 DNA stimulated cells to produce a significantly more *Cxcl10* mRNA, indicating that this sequence is more immunostimulatory than a control DNA sequence. The particular sequence within NRE1 found within poxvirus genomes is GA- rich (5' GAGAAAGAGAA 3') as such we have termed this the 'GAGA' sequence for simplicity.

Figure 3a Shows vaccinia virus strain Western Reserve (WR) protein C16 interacts with the Ku complex.

A cell line that expresses a tandem affinity purification (TAP) tagged version of the VACV WR C16 protein upon induction with doxycyclin was created using the Invitrogen HEK293 T-Rex system and the pcDNA-4 TO plasmid. C16 expression was induced by addition of doxycyclin (+C16) and the C16 protein was purified using sequential affinity purification with strepatactin and FLAG M2-agarose beads. The affinity purified C16 was then analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were stained with coomassie brilliant blue. The positions of molecular mass markers in kD are shown on the right.

C16 purified together with 2 proteins of approximately 70 and 80/86 kDa. These proteins were excised from the gel and were identified unequivocally by mass spectrometry as the Ku70 and Ku80/86 proteins. The Ku proteins are well characterised proteins that form a heterodimer and function in DNA repair and in the formation of antibody diversity by promoting VDJ recombination. Independent data showed that DNA-PK (a complex composed of DNA-protein kinase, Ku70 and Ku80/86) function as a sensor for cytoplasmic dsDNA leading to activation of innate immunity, we proposed that the VACV protein C16 is an inhibitor of this pathway. The ability of C16 to interact with Ku70 and Ku80/86 is consistent with this proposal.

Figure 3b Shows that when C16 is expressed at natural levels during vaccinia virus infection it binds to Ku80/86 and can be immunoprecipitated with anti-Ku80/86 antibody. The Ku80/86 antibody also precipitates the other components of the Ku complex.

Cells were infected with vaccinia virus (VACV) strain Western Reserve (WR) or with a VACV strain lacking the *C16L* gene (Fahy et al., JGV 2008) for 16 h or were mock infected (-). Cell lysates were prepared and the samples were either analysed by SDS-PAGE followed by immunoblotting with the indicated antibodies (left panel, WCL) or were immunoprecipitated with anti-Ku80/86 antibody (right panel). The immunoprecipitates were then analysed by SDS-PAGE and immunoblotting with antibodies against C16, Ku70 or DNA-PKcs. As a control an isotype matched mouse antibody (WR, ISO) was used in parallel. The molecular mass of the proteins detected is indicated on the left side in kDa.

Figure 4 Shows that VACV protein C16 inhibits the innate immune response of cells to dsDNA.

In the top panel: HEK293T cells were transfected with an IFNβ luciferase reporter plasmid and either empty vector (pcDNA3) or C16 in the pcDNA3 backbone. The next day, cells were stimulated with either 200 ng per well of a 96-well plate of poly(dA:dT), a synthetic B-DNA molecule, or were mock transfected. The cells were lysed at 6 h post transfection and the luciferase activity was measured compared to internal control. The presence of C16 resulted in a statistically significant reduction in the amount of luciferase production suggesting that C16 blocks DNA sensing.

Shown in the bottom panel: Murine embryo fibroblasts were transfected with either empty vector (EV) and DNA, or with a plasmid expressing C16 and DNA. The supernatant of cells was then tested for the chemokine Cxcl-10 by ELISA. The cells transfected with C16 and DNA produced significantly lower levels of Cxcl-10 protein than cells transfected with empty vector and DNA.

Figure 5 Shows that DNA-PK functions as a sensor for dsDNA leading to innate immunity.

Since Ku forms the heterotrimeric complex DNA-PK with the catalytic subunit (DNA-PKcs), knockout MEFs for Ku70, Ku80/86 and DNA-PKcs were obtained. The hypothesis that these molecules would be crucial to the innate immune response to DNA was confirmed by showing that these cells are deficient in the production of inflammatory chemokines and cytokines in response to DNA but not to other innate immune stimulants such as poly (I:C) and lipopolysaccharide (LPS).

Figure 6 Shows DNA-PK associates with cytoplasmic DNA.

Immunoblots of biotinylated-DNA/protein complexes precipitated with straptavidin from the cytoplasmic fraction of HEK293T cells at 0, 60 and 120 minutes following transfection of biotinylated DNA. All three components of DNA-PK; Ku70, Ku8/860 and DNA-PKcs, were found bound to cytoplasmic DNA. WCL, whole cell lysate. AP, affinity purification.

Figure 7 Shows upregulation of *DNA-PKcs*, *Ku70* and *Ku80/86* transcription following stimulation with dsDNA.

MEFs were stimulated by transfection with 10 μg/ml of dsDNA for the indicated times and the level of transcription of the indicated genes was measured by qPCR at the indicated times thereafter.

Figure 8 Shows that the length and concentration of dsDNA influences the activation of chemokine and cytokine transcription in fibroblasts.

a, Annealed complementary 45-bp oligonucleolides (An) become concatamerised into higher order DNA species (Con) following treatment with polynucleotide kinase and T4 DNA ligase. **b**, Transfection of these dsDNA concatamers into fibroblasts results in activation of *Il-6* and *Cxcl10* transcription in a dose-dependent manner. **c.** Stimulation of fibroblasts with dsDNA concatamers requires transfection of intact DNA into the cytoplasm. Pre-treatment of concatamers with DNase abrogates production of *Cxcl10* and *Il6* in response to DNA transfection. **d,** Stimulation of MEFs with dsDNA requires transfection since addition of dsDNA to the culture medium without complexing with transfection reagent fails to induce transcription of *Cxcl10*. **e**, Length dependency of dsDNA stimulation is shown by transfection of oligonucleotides of increasing sizes into MEFs and measurement of *Cxcl10* transcription.

Figure 9 Shows inflammatory cytokine expression in response to DNA is dependent on DNA-PKcs and Ku expression.

a, DNA transfection leads to the transcription of cytokines that increases over time and the expression of *Ccl5*, *Il6* and *Ccl4* is dependent on DNA-PKcs whereas *Cxcl2* expression in unaffected by the loss of this gene. No induction of *Ccl2, Ccl3, Il4, Il10* or *Ifng* was found following DNA stimulation. **b**, In adult murine fibroblasts the transcription of *Ifnb* in response to DNA, but not RNA, is perturbed in the absence of the Ku70 and Ku80/86. qPCR analysis of RNA harvested from wild type or Ku knockout cells 6 h after transfection with DNA (10 μg/ml) or Poly(I:C) (10 μg/ml).

Figure 10 Shows the innate immune response to DNA is (a) dependent on STING but (b) independent of toll-like receptor signalling, IPS-1, DAI, IRF5 and IRF7.

qPCR analysis of RNA harvested from MEFs of indicated genotypes 6 h after transfection with DNA (10 μg/ml) or Poly(I:C) (10 μg/ml) or infection with MVA (m.o.i. of 5).

Figure 11 shows a IRF3 does not localise to MVA DNA factories following infection in HeLa cells. Scale bar; 5 μm. **b** MVA replication, and **c** MVA protein synthesis, in MEFs (mouse embryonic fibroblasts)is unaffected by loss of Ku80/86.

Figure 12 Shows the innate immune response to HSV-1 infection is not dependent on DNA-PKcs. MEFs were infected with herpes simplex virus type 1 (HSV-1) (m.o.i of 1) for 6 h and the transcription of *Cxcl10* was analysed by qPCR.

Figure 13 Shows DNA-PK is expressed in fibroblasts but not primary macrophages. Immunoblotting of protein lysates (50 μg) from murine cells indicating the differential expression of DNA-PK components.

Figure 14 Shows DNA-PK interacts with IRF3 and activates an innate immune response. **a,** Transcription of *Cxcl10* and *Ifnb*, analysed by qPCR 6 h after DNA transfection, was abrogated in MEFs lacking *Irf3* when compared to wild type cells. **b,** The expression of *Cxcl10* in response to DNA transfection is dependent on TBK-1. **c**, IRF3 nuclear translocation is inhibited in DNA-PKcs-/- cells in response to DNA but not RNA, quantified in **d** (n=3, counts of at least 50 nuclei per slide). **e**, The stimulation of *Isg54* by dsDNA is dependent on IRF3 (left panel) and DNA-PKcs (right panel). *Isg54* transcription was measured by qPCR 6 h following DNA (10 μg/ml) or IFNβ (2000 U/ml) stimulation. **f**, Coimmunoprecipitation of IRF3 with DNA-PKcs and Ku8/860. Expression of IRF3 in HeLa cells followed by immunoprecipitation using either anti-IRF3 (left panel) or anti-Ku80/86 (right panel) and immunoblotting for indicated proteins. –ve: control IgG. ** $p<0.01$, * $p<0.05$, n=3, error bars +/- SEM. ns; non-stimulated. Scale bar; 5 μm.

Figure 15 Shows DNA-PK is important for the innate immune response to VACV (strain MVA) infection.

a, Ku70 and DNA-PKcs co-localise with VACV DNA factories by immunofluoresence. Cytoplasmic viral factories formed after MVA infection of HeLa cells and visualised with DAPI (labelled DAPI) co-localise with both Ku70 (left panel) and DNA-PKcs (right panel). Sites of co-localisation are indicated by white arrows, Scale bar; 5 μM. **b** and **c**, In DNA-PKcs-/- or Ku80-/- cells, cytokine transcription is impaired following MVA infection. qPCR analysis of *Cxcl10* and *Il6* transcription in DNA-PKcs-/- and Ku80-/- MEFs (mouse embryonic fibroblasts) following infection with increasing doses of MVA (left panels, representative of three independent experiments) or 6 h after infection, m.o.i.=5 (right panels). ** $p<0.01$, n=3, error bars +/- SEM, ni; non-infected.

Figure 16 Shows that deleting the C-terminal region of C16 does not abrogate the ability of C16 to inhibit innate immune sensing of dsDNA, whereas deleting N-terminal region does. Fibroblasts were transfected with empty vector (EV) or with the same plasmid containing the full length codon optimised C16L gene (C16FL), or a *C16L* gene encoding amino acids 1-203 (C16δC), or a *C16L* gene encoding amino acids 178-332 (C16δN). After 16 h the cell culture medium was collected and the presence of CXCL-10 was determined by ELISA.

Figure 17 Shows DNA-PK is critical for the innate immune response to DNA.

a, Various dsDNAs stimulate *Cxcl10* transcription when transfected into fibroblasts, as analysed by qPCR. **b**, In DNA-PKcs-/- cells, IFNβ and CXCL10 expression and *Ccl5* and *Il6* transcription are

abrogated in response to DNA, but not RNA transfection. Responses measured by ELISA or qPCR 6h after transfection. **c**, *Cxcl10* transcription increases over time in following DNA stimulation, but is absent in DNA-PKcs-/- MEFs (left panel). There is an unimpaired response to LPS treatment in these cells (right panel). **d**, In *Ku80-/-* cells the induction of *Cxcl10* and *Il6* is significantly impaired following DNA stimulation. *** p<0.001, ** p<0.01, * p<0.05, n=3, error bars +/- SEM, ns; nonstimulated. Data shown are representative of at least two separate experiments.

Figure 18a Shows DNA-PKcs kinase activity is not required for DNA sensing.

qPCR detection of CXCL10 transcription following stimulation of mouse embryonic fibroblasts from wild type (Balb/c) or DNA-PKcs kinase-mutant (SCID) mice with DNA.

Figure 18b Shows DNA-PKcs kinase activity is not required for DNA sensing *in vivo*.

qPCR detection of CXCL10 transcription 8 hours following injection of wild type (Balb/c) or DNA-PKcs kinase-mutant (SCID) mice with DNA, RNA or MVA. Lipofectamine 2000 (LF2K) injection was used as a negative control.

Figure 19 Shows virus lacking C16 induces greater amounts of specific inflammatory mediators

Groups of 5 BALB/c mice were infected wither either WR, knockout C16 virus (vΔC16) or revertant virus. A) BAL samples were taken at the specified timepoints and CXCL10 concentrations measured by ELISA. B.) BAL samples were taken at the specified timepoints and IL-6 protein concentrations were measured at the specified timepoints by ELISA. C) Tracheas from infected mice were harvested and IFNβ mRNA levels were assessed by qRT-PCR.

Detailed Description of the Disclosure

DNA-PK as employed herein is a complex composed of DNA-protein kinase, Ku70 and Ku80/86 which function as a sensor for cytoplasmic dsDNA leading to activation of innate immunity.

DNA sensor as employed herein refers to a sensor involved in detecting DNA, which may trigger an innate immune response that may influence the subsequent adaptive immune response.

Polynucleotide as employed herein is intended to refer to single or double stranded DNA.

dsDNA as employed herein is intended to refer to double stranded DNA.

Double stranded as employed herein refers to double stranded DNA including self-complementary loops, hairpin loops and the like.

DNA-PKcs as employed herein is an enzyme that in humans is encoded by the PRKDC gene. DNA-PKcs belongs to the phosphatidylinositol 3-kinase-related kinase protein family. The UniProt no for the human enzyme is P78527 and for the mouse enzyme is Q91UZ3. DNA-PKcs is the catalytic subunit of DNA-PK. On its own, DNA-PKcs is inactive. DNA-PKcs is required for the non-homologous end joining (NHEJ) pathway of DNA repair, which rejoins double-strand breaks. It is also required for VDJ recombination, a process that utilizes NHEJ to promote immune system diversity. DNA-PKcs knockout mice have severe combined immunodeficiency due to their V(D)J recombination defect.

VDJ recombinationis a mechanism of genetic recombination in the early stages of immunoglobulin (Ig) and T cell receptors (TCR) production of the immune system. VDJ recombination nearly-randomly combines Variable, Diverse, and Joining gene segments of vertebrates, and because of its randomness in choosing different genes, is able to diversely encode proteins to match antigens from bacteria, viruses, parasites and dysfunctional cells such as tumor cells.

Isolated polynucleotide as employed herein is intended to refer to polynucleotide isolated from its natural environment, for example isolated from the organism in which it naturally occurs (including isolated from the DNA of a virus in which it occurs) in a suitably pure form, or recombinantly prepared or chemically synthesised polynucleotide. Isolated would include *ex vivo* samples, as appropriate. The isolated DNA may comprise part of a molecule or entity in which it does not naturally occur, for example a recombinant virus, which is non-identical to a wild-type virus. In one embodiment the isolated polynucleotide is not inserted to or associated with any other genetic material.

Natural environment in the context of the present disclosure is intended to refer to a molecule or pathogen which contains the polynucleotide sequence in question without human intervention to insert the same.

Suitably pure form as employed herein is intended to refer to herein as polynucleotide sufficiently pure to perform the intended function, for example comprising no more than 50% additional matter, for example 40% or less, such as 30% or less, in particular 20% or less, especially 10% or less additional matter. Suitably pure as employed herein will extend to pure polynucleotide in a pure solvent, buffer or other carrier.

Consists essentially as employed herein is intended to refer to a polynucleotide sequence made up of at least 50% 'GAGA' repeats over its full length, for example 60%, ,70%, 80%, 90%, 95% or 99% 'GAGA' repeats.

Copy number as employed herein is intended to mean the number of occurrences of 'GAGA' in a polynucleotide.

Altered copy number as employed herein is intended to refer to a polynucleotide in which the wild type number of occurrences of GAGA is increased or decreased, or a recombinant polynucleotide in which GAGA has been engineered to occur a defined number of times, or a polynucleotide in which the occurrence of GAGA has been concentrated by excision of intervening non-GAGA bases. Low copy number as employed herein is intended to refer to a polynucleotide with less than 10% GAGA over its full length, such as less than 5%, such as less than 1%. Alternatively low copy number may be by reference to the absolute number of occurrences of the sequences, for example 5 or less, such as 4 or less, in particular 3 or less, especially 2 or less, including 0.

Rich in the motif as employed herein is intended to refer to a region comprising multiple occurrences of the relevant sequence, for example with an increased numbers of occurrences in comparison to what one would predict statically.

NRE1 as employed herein is intended to mean a sequence comprising sequence 5' GAGAAAGAGAA 3', for example as tandem repeats or separated by based pairs.

Ku80/86 as employed herein is intended to reflect the fact that the literature refers to the Ku complex forming component as Ku80 or Ku86. Nevertheless the present inventors believe that this is simply an inconsistency in the naming convention and that Ku 80 and Ku 86 are in fact the same entity.

C16 as employed herein is intended to refer to the protein of about 181 amino acids, derived from vaccinia virus, for example UniProt number P21100.

Each of the features [X] and [Y] are optional in the constructs of the invention in particular when the subscript of the bracket is 0.

In one embodiment the subscript for X is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50.

In one embodiment the subscript for Y is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50.

In one embodiment n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 or 149.

In one embodiment the polynucleotide sequence length comprises in the range 45 to 200 base pairs.

In one embodiment the polynucleotide sequence length comprises in the range 50 to 100 base pairs.

It is thought that the overall length of the polynucleotide contributes to the immunogenicity *in vivo*.

In one embodiment the polynucleotide is single stranded.

In one embodiment the 'GAGA' sequence is located at or towards the 5' end of a sequence.

In one embodiment the 'GAGA' sequence is located at or towards the 3' end of a sequence.

In one embodiment the 'GAGA' sequence is located at or approximately in the central region of a sequence.

In one embodiment the polynucleotide is double stranded, for example provided as individual strands or alternatively as a hairpin loop, self-complementary loops and the like.

In one embodiment the polynucleotide is circular.

In one embodiment the construct is linear DNA.

In one embodiment the polynucleotide further comprises one or more CpG motifs.

In one embodiment the polynucleotide or part thereof is palindromic.

In one embodiment the polynucleotide consists or consists essentially of 'GAGA' repeats, for example GAGAAAAGAGAAGAGAAAAGAGAA etc (SEQ ID NO: 3).

In a further embodiment the 'GAGA' repeats are tandem repeats, for example GAGAAAAGAGAAGAGAAAAGAGAA etc.

There is also provided a composition, such as pharmaceutically acceptable composition comprising a polynucleotide as defined herein.

In one embodiment the pharmaceutical composition is a vaccine composition further comprising an antigen, such a pathogen including an inactivated bacteria, an attenuated virus or

viral components, such as virus particles, or antigen, include a cancer antigen such a MAGE or the like and/or a self-antigen.

In some instances it is desirable to be able to break tolerance to self and induce an immune response to a self-antigen, for example those overexpressed by tumours/cancers. Often strong adjuvants are required to generate adequate immune responses.

In one embodiment the DNA molecules provided herein are adjuvants, for example suitable for generating or promoting a Th1 and/or Th2 type response to an antigen.

In one embodiment the DNA molecules and compositions of the disclosure are suitable for generally boosting or generating an innate immune response.

The adjuvant effect of the components described in the present disclosure can be employed in boosting the natural immune response *in vivo* to antigens, in particular an antigen derived from a pathogen. Thus in one embodiment the pharmaceutical formulation further comprises an antigen, in particular an antigen derived from a pathogen.

There remains a need for alternative adjuvants, in particular for use with antigens for which it is difficult to generate a protective antibody response thereto. Antigens that may benefit from improved adjuvants include: malaria antigens such as RTS,S described in WO93/10152 and derivatives thereof, for example as described in WO2009/080803; HIV antigens and tumour-associated antigens and derivatives thereof, such as MAGE see for example WO99/40188. The present disclosure provides in one or more embodiments an adjuvant that can be employed to generate an appropriate immune response, for example an antibody or T cell response to an antigen, for example a protective antibody response to an antigen derived from a pathogen.

In one or more aspects the adjuvants described herein may have application to cancer vaccines, in particular those where tolerance to self-antigens must be broken to obtain a therapeutically effective formulation, or those antigens from pathogens that induce tumour formation. Some examples of cancer antigens are given below.

Cancer Antigens

The first antigen which could be defined through its recognition by specific CTLs on autologous melanoma cells is termed MZ2-E (Van den Eynde, 1989) and is encoded by the gene MAGE-1 (Van der Bruggen, 1991). CTLs directed against MZ2-E recognise and lyse MZ2-E positive melanoma cells from autologous as well as from other patients provided that these cells have the HLA.A1 allele.

The MAGE-1 gene belongs to a family of 12 closely related genes, MAGE 1, MAGE 2, MAGE 3, MAGE 4, MAGE 5, MAGE 6, MAGE 7, MAGE 8, MAGE 9, MAGE 10, MAGE 11, MAGE 12, located on chromosome X and sharing with each other 64 to 85% homology in their coding sequence (De Plaen, 1994). These are sometimes known as MAGE Al, MAGE A2, MAGE A3, MAGE A4, MAGE A5, MAGE A6, MAGE A7, MAGE A8, MAGE A9, MAGE A 10, MAGE A 11, MAGE A 12 (The MAGE A family). Two other groups of proteins are also part of the MAGE family although more distantly related. These are the MAGE B and MAGE C group. The MAGE B family includes MAGE B1 (also known as MAGE Xpl, and DAM 10), MAGE B2 (also known as MAGE Xp2 and DAM 6) MAGE B3 and MAGE B4 - the Mage C family currently includes MAGE C1 and MAGE C2. In general terms, a MAGE protein can be defined as containing a core sequence signature located towards the C-terminal end of the protein

(for example with respect to MAGE A1 a 309 amino acid protein, the core signature corresponds to amino acid 195-279).

Below is a list antigens derived from various pathogens.

Malaria Antigens

Malaria antigens include those derived from pathogens, such as a Plasmodium sp. eg P. falciparum or P. vivax. For example, antigens derived from P. falciparum include circumsporozoite protein (CS protein), PfEMP-1 , Pfs 16 antigen, MSP-1 , MSP-3, LSA-1 , LSA-3, AMA-1 and TRAP. A particular hybrid antigen that may be mentioned is RTS. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of P.falciparum linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS, S The structure or RTS and RTS, S is disclosed in WO93/10152. TRAP antigens are described in WO90/01496.

Other Plasmodium antigens include P. falciparum EBA, GLURP, RAP1 , RAP2, Sequestrin, Pf332, STARP, SALSA, PfEXPI , Pfs25, Pfs28, PFS27/25, Pfs48/45, Pfs230 and their analogues in other Plasmodium spp.

Possible antigens from *P. vivax* include circumsporozoite protein (CS protein) and Duffy antigen binding protein and immunogenic fragments thereof, such as PvRII (see eg WO02/12292).

Antigens from other Pathogens.

Suitable polypeptide antigens to be administered as polypeptide or polynucleotide encoding polypeptide according to the invention include antigens derived from HIV (eg HIV-1), human herpes viruses, cytomegalovirus, especially Human, (such as gB or derivatives thereof), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpl, II, III and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen, PreS1, PreS2 and Surface env proteins, Hepatitis B core antigen or pol), hepatitis C virus (eg Core, E1 , E2, P7, NS2, NS3, NS4A, NS4B, NS5A and B) and hepatitis E virus antigen, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), or antigens from parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 1 1 , 16, 18, eg L1 , L2, E1 , E2, E3, E4, E5, E6, E7), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (such as haemaggluttin, nucleoprotein, NA, or M proteins, or combinations thereof), or antigens derived from bacterial pathogens such as Neisseria spp, including N. gonorrhea and N. meningitidis, eg, transferrin-binding proteins, lactoferrin binding proteins, PiIC, adhesins); S. pyogenes (for example M proteins or fragments thereof, C5A protease), S. agalactiae, S. mutans; H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella catarrhalis (for example high and low molecular weight adhesins and invasinsj; Bordetella spp, including B. pertussis (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis, M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp, including L pneumophila; Escherichia spp, including enterotoxic E. coli (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic E. coli,

enteropathogenic E. coli (for example shiga toxin- like toxin or derivatives thereof); Vibrio spp, including V. cholera (for example cholera toxin or derivatives thereof; Shigella spp, including S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp, including Y. enterocolitica (for example a Yop protein) , Y. pestis, Y. pseudotuberculosis; Campylobacter spp, including C. jejuni (for example toxins, adhesins and invasins) and C. coli; Salmonella spp, including S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp., including L monocytogenes; Helicobacter spp, including H. pylori (for example urease, catalase, vacuolating toxin); Pseudomonas spp, including P. aeruginosa; Staphylococcus spp., including S. aureus, S. epidermidis; Enterococcus spp., including E. faecalis, E. faecium; Clostridium spp., including C. tetani (for example tetanus toxin and derivative thereof), C. botulinum (for example botulinum toxin and derivative thereof,), C. difficile (for example Clostridium toxins A or B and derivatives thereof); Bacillus spp., including B. anthracis (for example anthrax toxinand derivatives thereof); Corynebacterium spp., including C. diphtheriae (for example diphtheria toxin and derivatives thereof); Borrelia spp., including B. burgdorferi (for example OspA, OspC, DbpA, DbpB), B. garinii (for example OspA, OspC, DbpA, DbpB), B. afzelii (for example OspA, OspC, DbpA, DbpBJ, B. andersonii (for example OspA, OspC, DbpA, DbpB), B. hermsii; Ehrlichia spp., including E. equi and the agent of the Human Granulocytic Ehrlichiosis; Rickettsia spp, including R. rickettsii; Chlamydia spp., including C. trachomatis, C. pneumoniae, C. psittaci; Leptospira spp., including L. interrogans; Treponema spp., including T. pallidum (for example the rare outer membrane proteins), T. denticola, T. hyodysenteriae; or derived from parasites such as Plasmodium spp., including P. falciparum and P. vivax; Toxoplasma spp., including T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp., including E. histolytica; Babesia spp., including B. microti; Trypanosoma spp., including T. cruzi; Giardia spp., including G. lamblia; leishmania spp., including L. major; Pneumocystis spp., including P. carinii; Trichomonas spp., including T. vaginalis; Schisostoma spp., including S. mansoni, or derived from yeast such as Candida spp., including C. albicans; Cryptococcus spp., including C. neoformans.

Further bacterial antigens include antigens derived from Streptococcus spp, including S. pneumoniae (PsaA, PspA, streptolysin, choline-binding proteins) and the protein antigen Pneumolysin (Biochem Biophys Acta, 1989, 67, 1007; Rubins et al., Microbial Pathogenesis, 25, 337-342), and mutant detoxified derivatives thereof (WO90/06951; WO99/03884). Other bacterial antigens include antigens derived from Haemophilus spp., including H. influenzae type B (for example PRP and conjugates thereof), non typeable H. influenzae, for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464) or multiple copy variants or fusion proteins thereof.

In particular, the methods or compositions of the present invention may be used to protect against or treat viral disorders such as those caused by Hepatitis B virus, Hepatitis C virus, Human papilloma virus, Human immunodeficiency virus (HIV), or Herpes simplex virus; bacterial diseases such as those caused by Mycobacterium tuberculosis (TB) or Chlamydia sp; and protozoal infections such as malaria, for example by immunization which an appropriate antigen and an adjuvant according to the present invention.

It is to be recognised that these specific disease states, pathogens and antigens have been referred to by way of example only, and are not intended to be limiting upon the scope of the present invention.

The pathogen may, for example, be Mycobacterium tuberculosis. Exemplary antigens derived from M. tuberculosis are for example alpha- crystallin (HspX), HBHA, Rv1753, Rv2386, Rv2707, Rv2557, Rv2558, RPFs: RvO837c, Rv1884c, Rv2389c, Rv2450, Rv1009, aceA (RvO467), ESAT6, Tb38-1 , Ag85A, -B or -C, MPT 44, MPT59, MPT45, HSP10, HSP65, HSP70, HSP 75, HSP90, PPD 19kDa [Rv3763], PPD, 38kDa [RvO934]), PstS1 , (RvO932), SodA (Rv3846), Rv2031 c, 16kDa, Ra12, TbH9, Ra35, Tb38-1 , Erd 14, DPV, MTI, MSL, DPPD, mTCC1 , mTCC2, hTCC1 (WO 99/51748) and hTCC2, and especially Mtb32a, Ra35, Ra12, DPV, MSL, MTI, Tb38-1 , mTCC1 , TbH9 (Mtb39a), hTCC1 , mTCC2 and DPPD. Antigens derived from M. tuberculosis also include fusion proteins and variants thereof where at least two, or for example, three polypeptides of M. tuberculosis are fused into a larger protein. Such fusions may comprise or consist of Ra12-TbH9-Ra35, Erd14-DPV-MTI, DPV-MTI-MSL, Erd14-DPV-MTI-MSL- mTCC2, Erd14-DPV-MTI-MSL, DPV-MTI-MSL-mTCC2, TbH9-DPV-MTI (WO 99/51748), Ra12-Tbh9-Ra35-Ag85B and Ra12-Tbh9-Ra35- mTCC2. A particular Ra12-Tbh9-Ra35 sequence that may be mentioned is defined by SEQ ID No 6 of WO2006/117240 together with variants in which Ser 704 of that sequence is mutated to other than serine, eg to Ala, and derivatives thereof incorporating an N- terminal His tag of an appropriate length (eg SEQ ID No 2 or 4 of WO 2006/117240) or a sequence containing an optional starting M and an optional N-terminal His-His tag (positions 2 and 3) and in which the Ala mutated relative to the wild-type Ser is at position 706.
The pathogen may, for example, be a Chlamydia sp. eg C. trachomatis. Exemplary antigens derived from Chlamydia sp eg C. trachomatis are selected from CT858, CT089, CT875, MOMP, CT622, PmpD, PmpG and fragments thereof, SWIB and immunogenic fragments of any one thereof (such as PmpDpd and PmpGpd) and combinations thereof. Suitable combinations of antigens include CT858, CT089 and CT875. Specific sequences and combinations that may be employed are described in WO2006/104890.

The adjuvant effect of the present disclosure provides a general boosting of the immune response, in particular innate immune responses which may assist in generating a strong adaptive immune response to specific antigens via the development of antibodies (Th2 type immune response) or T cell response (Th1 type immune response) to said antigens. The antigens may, for example include antigens such as surface or internal proteins from viruses, bacteria, fungi or parasites .

HIV-1 derived antigens for use in the invention may thus for example be selected from Gag (for example full length Gag), p17 (a portion of Gag), p24 (another portion of Gag), p41 , p40, Pol (for example full length Pol), RT (a portion of Pol), p51 (a portion of RT), integrase (a portion of Pol), protease (a portion of Pol), Env, gp120, gp140 or gp160, gp41, Nef, Vif, Vpr, Vpu, Rev, Tat and immunogenic derivatives thereof and immunogenic fragments thereof, particularly Env, Gag, Nef and Pol and immunogenic derivatives thereof and immunogenic fragments thereof including p17, p24, RT and integrase. HIV vaccines may comprise polypeptides and/or polynucleotides encoding polypeptides corresponding to multiple different HIV antigens for example 2 or 3 or 4 or more HIV

antigens which may be selected from the above list. Several different antigens may, for example, be comprised in a single fusion protein.

For example an antigen may comprise Gag or an immunogenic derivative or immunogenic fragment thereof, fused to RT or an immunogenic derivative or immunogenic fragment thereof, fused to Nef or an immunogenic derivative or immunogenic fragment thereof wherein the Gag portion of the fusion protein is present at the 5' terminus end of the polypeptide. A particular example of a Gag sequence for use in the invention comprises p17 and/or p24 encoding sequences.
A RT sequence may contain a mutation to substantially inactivate any reverse transcriptase activity (see WO03/025003). The RT gene is a component of the bigger pol gene in the HIV genome. It will be understood that the RT sequence employed according to the invention may be present in the context of Pol, or a fragment of Pol corresponding at least to RT. Such fragments of Pol retain major CTL epitopes of Pol. In one specific example, RT is included as just the p51 or just the p66 fragment of RT.

Optionally the Nef sequence for use in the invention is truncated to remove the sequence encoding the N terminal region i.e. removal of from 30 to 85 amino acids, for example from 60 to 85 amino acids, particularly the N terminal 65 amino acids (the latter truncation is referred to herein as trNef).

The Env antigen may be present in its full length as gp160 or truncated as gp140 or shorter (optionally with a suitable mutation to destroy the cleavage site motif between gp120 and gp41). The Env antigen may also be present in its naturally occurring processed form as gp120 and gp41. These two derivatives of gp160 may be used individually or together as a combination.
One embodiment of the invention employs an immunogenic polypeptide containing p17, p24 Gag, p66 RT, truncated Nef (devoid of nucleotides encoding terminal amino-acids 1-85 - "trNef") in the order Gag, RT, Nef. In polynucleotides encoding immunogenic polypeptides, suitably the p24 Gag and p66 RT are codon optimized. Specific polynucleotide sequences and corresponding polypeptide antigens employed in the invention include:

p17, p24 Gag - p66 RT - truncated Nef;	truncated Nef - p66 RT - p17, p24 Gag;
truncated Nef - p17, p24 Gag - p66 RT;	p66 RT - p17, p24 Gag - truncated Nef;
p66 RT - truncated Nef - p17, p24 Gag;	p17, p24 Gag - truncated Nef - p66 RT.

An exemplary fusion is a fusion of Gag, RT and Nef particularly in the order Gag-RT-Nef. Another exemplary fusion is a fusion of p17, p24, RT and Nef particularly in the order p24-RT-Nef-p17 (referred to elsewhere herein as "F4").

In another embodiment an immunogenic polypeptide contains Gag, RT, integrase and Nef, especially in the order Gag-RT-integrase-Nef.

In other embodiments the HIV antigen may be a fusion polypeptide which comprises Nef or an immunogenic derivative thereof or an immunogenic fragment thereof, and p17 Gag and/or p24 Gag or immunogenic derivatives thereof or immunogenic fragments thereof, wherein when both p17 and p24 Gag are present there is at least one HIV antigen or immunogenic fragment between them.

For example, Nef is suitably full length Nef.

In one embodiment an immunogenic polypeptide comprises both p17 and p24 Gag or immunogenic fragments thereof. In such a construct the p24 Gag component and p17 Gag component are separated by at least one further HIV antigen or immunogenic fragment, such as Nef and/or RT or immunogenic derivatives thereof or immunogenic fragments thereof. See WO 2006/013106 for further details.

Some nucleotide sequences according to the invention encode the following:

p24 - RT - Nef - p17;	p24 - RT<*> - Nef - p17;
p24-p51RT-Nef-p17;	p24-p51RT<*>- Nef-p17;
p17-p51RT-Nef;	p17-p51RT<*>- Nef;
Nef-p17;	Nef-p17 with linker;
p17- Nef;	p17 - Nef with linker;

<*> represents RT methionine592 mutation to lysine

HIV antigens employed in the present invention may be derived from any HIV clade, for example clade A, clade B or clade C. For example the HIV antigens may be derived from clade A or B, especially B.

The aforementioned antigens may be employed in the form of immunogenic derivatives or immunogenic fragments thereof rather than the whole antigen. As used herein the term "immunogenic derivative" in relation to an antigen of native origin refers to an antigen that may have been modified in a limited way relative to its native counterparts. For example it may include a point mutation which may change the properties of the protein, e.g. by improving expression in prokaryotic systems or by removing undesirable activity, e.g. enzymatic activity. Immunogenic derivatives will however be sufficiently similar to the native antigens such that they retain their antigenic properties and remain capable of raising an immune response against the native antigen. Whether or not a given derivative raises such an immune response may be measured by a suitably immunological assay such as an ELISA (for antibody responses) or flow cytometry using suitable staining for cellular markers (for cellular responses).

Immunogenic fragments are fragments which encode at least one epitope, for example a CTL epitope, typically a peptide of at least 8 amino acids. Fragments of at least 8, for example 8 to 10 amino acids or up to 20, 50, 60, 70, 100, 150 or 200 amino acids in length are considered to fall within the intended meaning as long as the polypeptide demonstrates antigenicity, that is to say that the major epitopes (eg CTL epitopes) are retained by the polypeptide.

In one embodiment the antigen employed and the sequence according to the invention are linked, for example conjugated or may be expressed together.

In one embodiment antigens employed in the present disclosure may be delivered *in vivo* by viral vectors, using known technology where the viral vectors encoding said antigens and are designed to express them *in vivo*.

The viral vector of the invention may be replication defective. This means that it has a reduced ability to replicate in non-complementing cells and/or viruses from other species that are unable to replicate in the species being immunised, compared to the wild type virus. This may be

brought about by mutating the virus e.g. by deleting a gene involved in replication, for example in adenoviral vectors the E1 or E2 region.

In one embodiment a polynucleotide according to the present disclosure is delivered *in vivo* by a viral vector, for example comprising the sequence and/or suitable for replicating the sequence *in vivo*. The vector may further encode an antigen as described above.

The viral vectors can be produced on any suitable cell line in which the virus is capable of replication. Where the virus has impaired replication due to missing factors, then complementing cell lines which provide the factors missing from the viral vector that result in its impaired replication characteristics can be used.

In one or more embodiments the adjuvant compositions, immunogenic compositions and/or vaccine compositions described herein may comprise one or more further known adjuvants (such as one, two or three further adjuvants).

In an embodiment the adjuvant is a Toll like receptor (TLR) 4 ligand, for example an agonist such as a lipid A derivative particularly monophosphoryl lipid A or more particularly 3-deacylated monophoshoryl lipid A (3D - MPL).

3-Deacylated monophosphoryl lipid A is known from US patent No. 4,912,094 and UK patent application No. 2,220,211 (Ribi) and is available from Ribi Immunochem, Montana, USA and old under the trademark MPL(R) by Corixa corporation. 3D-MPL primarily promotes CD4+ T cell responses with an IFN-g (Th1) phenotype. It can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Suitably in the compositions of the present invention small particle 3D- MPL may be used. Small particle 3D-MPL has a particle size such that it may be sterile-filtered through a 0.2 micron filter. Such preparations are described in WO94/21292. Synthetic derivatives of lipid A are known and thought to be TLR 4 agonists.

Another immunostimulant for use in formulations of the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree Quilaja Saponaria Molina and was first described as having adjuvant activity by Dalsgaard et al. in 1974 ("Saponin adjuvants", Archiv. fur die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254). Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of Quillaja saponaria Molina which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response. Particular formulations of QS21 have been described which further comprise a sterol (WO 96/33739). The ratio of QS21: sterol will typically be in the order of 1 : 100 to 1 : 1 weight to weight. Generally an excess of sterol is present, the ratio of QS21 : sterol being at least 1 : 2 w/w. Typically for human administration QS21 and sterol will be present in a vaccine in the range of about 1 µg to about 100 µg, such as about 10 µg to about 50 µg per dose.

Liposomes generally contain a neutral lipid, for example phosphatidylcholine, which is usually non-crystalline at room temperature, for example eggyolk phosphatidylcholine, dioleoyl phosphatidylcholine or dilauryl phosphatidylcholine. Liposomes may also contain a charged lipid which when QS21 is present may increase the stability of lipsome-QS21 structures for liposomes

composed of saturated lipids. In these cases the amount of charged lipid is often 1-20% w/w, such as 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), such as 20-25%. The saponins may be separate in the form of micelles, mixed micelles (generally, but not exclusively with bile salts) or may be in the form of ISCOM matrices (EP 0 109 942), liposomes or related colloidal structures such as worm-like or ring-like multimeric complexes or lipidic/layered structures and lamellae when formulated with cholesterol and lipid, or in the form of an oil in water emulsion (for example as in WO95/17210).

In one aspect the adjuvant comprises 3D-MPL.

In one aspect the adjuvant comprises QS21.

In one aspect the adjuvant comprises CpG.

In one aspect the adjuvant is formulated as an oil in water emulsion.

In one aspect the adjuvant is formulated as liposomes.

Adjuvants combinations include 3D-MPL and QS21 (EP0671948), oil in water emulsions comprising 3D-MPL and QS21 (WO95/17210, WO98/56414), 3D-MPL formulated with other carriers (EP0689454) or 3D-MPL and QS21 in a liposomal formulation. Other suitable adjuvant systems comprise a combination of 3D-MPL, QS21 and a CpG oligonucleotide as described in US6558670 and US6544518.

In one embodiment the molecules and compositions of the disclosure have antiviral properties, for example in that they stimulate the immune system to fight invading viruses

In one embodiment there is provided a vector, for example a viral vector such as an adenoviral vector comprising a polyncucleotide according to the present disclosure. These may be administered to patients and once *in vivo* the vector may replicate the polynucleotide. Alternatively the viral vectors may be replication deficient and may simply represent an alternative way of presenting the construct to the immune system.

Viral vectors may be derived from any suitable viral type. Virus types include: -

-dsDNA viruses (e.g. Adenoviruses, Herpesviruses, Poxviruses)

- ssDNA viruses (+) sense DNA (e.g. Parvoviruses)

- dsDNA-RT viruses (e.g. Hepadnaviruses) DNA virus types include: Adenoviridae; Papillomaviridae; Parvoviridae; Herpesviridae eg Herpes simplex virus type 1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus and other human herpes viruses; Poxviridae such as the orthopoxviruses vaccinia virus, cowpox virus, horsepox virus, rabbitpox virus, camelpox virus, ectromelia virus: other poxviruses from chordates, such as members of the avipox, leporipox, suipox, capripox, yatapox, parapox, and molluscipox virus genera; deerpox virus, squirrelpox virus and crocodile poxvirus; Hepadnaviridae eg Hepatitis B virus; Polyomaviridae eg Polyoma virus, JC virus (progressive multifocal leucoencephalopathy); Circoviridae eg Transfusion Transmitted Virus.

The viral vector may be, by way of example, a single stranded DNA virus belonging to Parvoviridae such as adeno-associated virus.

The viral vector may be, by way of example, a double stranded DNA virus belonging to Herpesviridae such as Epstein - Barr virus, herpes simplex virus (HSV); Poxviridae such as the orthopoxviruses vaccinia virus (including different strains and derivatives such as modified virus Ankara, MVA), cowpox virus, horsepox virus, rabbitpox virus, camelpox virus, ectromelia virus: other

poxviruses from chordates, such as members of the avipox, leporipox, suipox, capripox, yatapox, parapox, and molluscipox genera; deerpox virus, squirrelpox virus and crocodile poxvirus;.

In one or more embodiments the viral vector employed is avirulent.

In one embodiment the modified viruses that do not express a C16, or other proteins, that inhibit innate immune sensing of DNA by DNA-PK, thereby the immunogenicity of the virus can be optimised.

In this embodiment the viral vector can be considered to be an alternative presentation of the polynucleotide sequence to the immune system, by for example being engineered to comprise sufficient copies of the GAGA sequence to stimulate an immune response.

The number of copies of GAGA is a virus may be increased by repeated passaging in an appropriate cell culture.

In one embodiment a viral vector is engineered to have zero or a reduced copy number of the GAGA sequence, such that the vector has low immunogenicity *in vivo*.

In one embodiment a viral vector according to the present disclosure is provided as a pharmaceutical composition.

In one embodiment there is provided a host comprising a sequence described herein. The host may, for example be suitable for synthesising a recombinant sequence according to the disclosure *in vitro*.

In one embodiment there is provided a method of generating or boosting an innate immune response comprising the step of administering a therapeutically effective amount of a polynucleotide, vector or a composition as defined herein.

In one embodiment the boosting of an immune response, such as an innate immune response leading to an adaptive immune response *in vivo*, particularly in a human or animal, especially in association with treatment or prophylaxis.

It may be useful to boost the innate and adaptive immune response in certain populations of patients, for example those with immune deficiency, such cancer patients, HIV patients and the like. Prophylactic treatment may be prior to or shortly after exposure to a virus, for example influenza, HIV, RSV, HRV, HPV, HCV or similar.

In one embodiment there is provided *ex vivo* or *in vitro* use of any suitable aspect of the disclosure as described herein to generate and innate immune response.

In one embodiment there is provided a method of modulating immunogenicity of a polynucleotide by inserting or deleting a sequence comprising one or more copies of the “GAGA” polynucleotide, for example by introducing and/or increasing the number of copies of the GAGA sequence in the polynucleotide, for example virus genetic material, such as the virus genome thereby increasing the immunogenicity of a virus.

It may be useful to increase the immunogenicity of a virus when, for example it is to be employed in vaccination, for example prophylactic vaccination because this may raise the visibility of the modified viruses to the immune system. In some instances, for example when pathogens such as a viruses or components thereof are employed in vaccines as the antigenic component, it is desirable to have a relatively high immunogenicity.

Decreasing or eliminating the copies of the GAGA sequence in the virus genetic material, such as the virus genome decreases the immunogeneticity of the virus. This may, for example be useful when the virus is to be employed in gene therapy. Thus, in other instances, for example when viruses are employed in gene therapy to deliver agents *in vivo*, then it is desirable to employ a virus with low immunogenicity. The virus according to this aspect of the invention may optionally have genes which encode C16, or similar proteins that inhibit sensing of DNA by DNA-PK, silenced or deleted.

In one embodiment a therapeutic virus, for example a replication competent virus such an oncolytic virus (WO2005/118825), is engineered to have zero or a reduced copy number of the GAGA sequence, such that the virus has low immunogenicity *in vivo.*

Low immunogenicity of a virus or viral vector, may increase the *in vivo* half-life allowing a longer residency time for generating a therapeutic effect.

Modification of the immunogenicity of the viruses employing the methods described herein are suitable for DNA viruses, including double stranded and single stranded viruses.

The present method allows the use of known techniques to manipulate the immunogenic properties of viruses, which is very useful in the field therapeutic and prophylactic agents.

Thus in one embodiment there is provided a method of genetically engineering a virus with an altered GAGA copy number.

In one embodiment there is provided a method of genetically engineering a virus wherein the GAGA copy number is increased.

In one embodiment there is provided a method of genetically engineering a virus wherein the GAGA copy number is decreased, for example is low or zero.

In one embodiment there is provided a genetically engineered virus with an altered GAGA copy number.

In one embodiment there is provided a polynucleotide, for example comprised in a virus obtained or obtainable by a process described herein.

In one embodiment there is provided use of an entity described herein for use in treatment or prophylaxis, for example for use as an adjuvant.

In one embodiment there is provided *ex vivo* or *in vitro* use of an isolated polynucleotide sequence comprising a region rich in the motif GAGA for example rich in the motif $([X]_{0-50}GAGAAAGAGAA[Y]_{0-50})_n$ wherein n is an integer 1 to 100, X is independently selected from a base and Y is independently selected from a base, to generate an innate immune response.

In one embodiment there is provided use of DNA-PK as a DNA sensor.

In one embodiment there is provided a method, for example an *in vitro* method of using DNA-PK as a DNA sensor.

In one embodiment there is provided a method of stimulating an innate immune response through DNA-PK signalling.

In one embodiment there is provided a chemical entity, polypeptide or protein which inhibits the stimulation of innate immunity by DNA-PK, for example by prevention of signalling therefrom or by destabilisation of the complex or prevention of the formation of the same.

In one embodiment there is provided a chemical entity, polypeptide or protein which inhibits the stimulation of innate immunity through or by the Ku complex.

In one embodiment a mutated Ku complex, is used as a dominant negative, for example a mutation is introduced that prevents downstream signalling and/or binding to DNA-PKcs.

In a further embodiment there is provided an isolated polypeptide C16 or a fragment thereof.

In one embodiment there is provided C16 or an active fragment thereof for use in therapy, in particular for inhibiting stimulation of innate immune response, for example an innate immune response is mediated by DNA-PK.

In one embodiment there is provided C16 for use as an inhibitor of DNA-PK.

Whilst not wishing to be bound by theory it is thought that this is the first time that a mechanism involving DNA-PK in the stimulation of the innate immune system has been disclosed.

In another aspect the present disclosures provide isolated C16, for example for use in down regulating innate immune responses, in particular inappropriate immune responses, for example those generated in autoimmune diseases such as Crohn's disease, Inflammatory bowel disease, rheumatoid arthritis, Sjogren's syndrome, multiple sclerosis, Alopecia areata, Ankylosing Spondylitis, Coeliac Disease, Dermatomyositis, Diabetes mellitus type 1, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's thyroiditis, Idiopathic thrombocytopenic purpura, Lupus erythematosus, Mixed Connective Tissue Disease, Myasthenia gravis, Narcolepsy, Pemphigus vulgaris, Pernicious anaemia, Psoriasis, Psoriatic Arthritis, Polymyositis, Primary biliary cirrhosis, Relapsing polychondritis, Temporal arteritis, Scleroderma, Ulcerative Colitis, Vasculitis and Wegener's granulomatosis.

In another aspect of the present disclosure C16 can be provided as an active N-terminal fragment consisting of amino-acid residues 1-203 of the protein.

In one embodiment there is provided a compositions (such as a pharmaceutical composition) comprising C16 or an active fragment thereof (for example the N-terminus) for use in treatment.

The first 203 amino acids constitute the N-terminal fragment of C16. The sequence is as follows:

MDIYDDKGLQ TIKLFNNEFD CIRNDIRELF KHVTDSDSIQ LPMEDNSDII ENIRKILYRR LKNVECVDI D STITFMKYDPNDDNKRTCSN WVPLTNNYME YCLVIYLETP ICGGKIKLYH PTGNIKSDKD IMFAKTLD FK SKKVLTGRKT IAVLDISVSYNRSMTTIHYN DDVDIDIHTD KNGKELCYCY ITIDDHYLVD VET SEQ ID NO: 4

In one aspect the invention extends to the N-terminus of C16.

In one embodiment there is provided use of a virus wherein the gene encoding the protein C16 is silenced or deleted, said virus comprising a 'GAGA' sequence (for example a naturally occurring sequence or an engineered sequence) wherein the virus is suitable for use in upregulation of an innate immune response as mediated through a DNA-PK complex.

In one embodiment there is provided a method of screening for modulators of innate immunity comprising the step; admixing the potential modulator with an active form of DNA-PK or components comprising the same, optionally in the presence of a control; and, measuring the kinase activity of the complex via its ability to phosphorylate a specific peptide substrate.

A second method is also provided wherein, the potential modulator, optionally in the presence of a control, may be added to cells expressing the active DNA-PK components and the secreted levels of one or more inflammatory cytokines, chemokines or interferons for example, selected from CXCL-10, IL-6, CCL-5 or IFN-β, measured.

In one embodiment there is provided chemical inhibitors identified employing the said method.

In one embodiment there is provided a kit comprising an isolated polynucleotide sequence comprising a region rich in the motif GAGA.

In one embodiment there is provided a kit comprising C16 or an active fragment thereof.

It is also envisaged that more than one embodiment described herein may be combined, as technically appropriate.

In the context of this specification "comprising" is to be interpreted as "including".

Aspects of the invention comprising certain elements are also intended to extend to alternative embodiments "consisting" or "consisting essentially" of the relevant elements.

EXAMPLES

Example 1

Double stranded DNA oligonucleotides (100 bp) containing control sequence (ISD), 50 bp control sequence and 50 bp of the GAGA sequence repeated (50% GAGA) and 100 bp of GAGA (100% GAGA) were transfected into cells and the upregulation of the CXCL-10 gene transcription was measured using real time reverse-transcription-PCR.

Data shown in Figure 1 demonstrate that the GAGA sequence increases the potency of the stimulation of cells compared with a control DNA sequence.

Example 2

The DNA target sequence for Ku (NRE1) is immunostimulatory.

It has been previously reported that Ku binds preferentially to the NRE1 sequence in the murine mammary tumour virus.

Interestingly, a sequence within the NRE1 sequence is also found in variable copy number in poxvirus genomes as well as the genomes of other DNA viruses.

It was proposed that since Ku binds the NRE1 DNA sequence preferentially and that Ku activates the immune system in response to DNA, the NRE1 sequence might be particularly immunostimulatory.

Stimulation of MEFs with either a control (ISD) DNA or NRE1 DNA lead to the observation that NRE1 DNA stimulated cells produce significantly more *Cxcl10* mRNA, indicating that this sequence is more immunostimulatory than a control DNA sequence. See Figure 2

Example 3a

Vaccinia virus strain Western Reserve (WR) protein C16 interacts with the Ku complex.

A cell line that expresses a tandem affinity purification (TAP) tagged version of the VACV WR C16 protein upon induction with doxycyclin was created using the Invitrogen HEK293 T-Rex system and the pcDNA-4 TO plasmid. C16 expression was induced by addition of doxycyclin (+C16) and the C16 protein was purified using sequential affinity purification with strepatactin and FLAG M2-agarose beads. The affinity purified C16 was then analysed by SDS-polyacrylamide gel electrophoresis (SDS-

PAGE) and proteins were stained with coomassie brilliant blue. The positions of molecular mass markers in kiloDaltons are shown on the right.

C16 purified together with 2 proteins of approximately 70 and 80/86 kDa. These proteins were excised from the gel and were identified unequivocally by mass spectrometry as the Ku70 and Ku80/86 proteins. The Ku proteins are well characterised proteins that form a heterodimer and function in DNA repair and in the formation of antibody diversity by promoting VDJ recombination.

Independent data showed that DNA-PK (a complex composed of DNA-protein kinase, Ku70 and Ku80/86) function as a sensor for cytoplasmic dsDNA leading to activation of innate immunity, we proposed that the VACV protein C16 is an inhibitor of this pathway. The ability of C16 to interact with Ku70 and Ku80/86 is consistent with this proposal. See Figure 3a.

Example 3b

Cells were infected with vaccinia virus strain Western Reserve (WR) or with a vaccinia virus strain lacking the *C16L* gene (Fahy et al., JGV 2008) for 16 h or were mock infected (-). Cell lysates were prepared and the samples were either analysed by SDS-PAGE followed by immunoblotting with the indicated antibodies (left panel, WCL) or were immunoprecipitated with anti-Ku80/86 antibody (right panel). The immunoprecipitates were then analysed by SDS-PAGE and immunoblotting with antibodies against C16, Ku70 or DNA-PKcs. As a control, an isotype matched mouse antibody (WR, ISO) was used in parallel. The molecular mass of the proteins detected is indicated on the left side in kDa.

The figure shows that when C16 is expressed at natural levels during VACV infection it binds to Ku80/86 and can be immunoprecipitated with anti-Ku80/86 antibody. The Ku80/86 antibody also precipitates the other components of the Ku complex. See Figure 3b.

Example 4

Shows that VACV protein C16 inhibits the innate immune response of cells to dsDNA.

In the top panel: HEK293T cells were transfected with an IFNβ luciferase reporter plasmid and either empty vector (pcDNA3) or C16 in the pcDNA3 backbone. The next day, cells were stimulated with either 200 ng per well of a 96-well plate of poly(dA:dT), a synthetic B-DNA molecule, or were mock transfected. The cells were lysed at 6 h post transfection and the luciferase activity was measured compared to internal control. The presence of C16 resulted in a statistically significant reduction in the amount of luciferase production suggesting that C16 blocks DNA sensing.

Fig 4 Bottom panel: Murine embryo fibroblasts were transfected with either empty vector and DNA or with a plasmid expressing C16 and DNA. The supernatant of cells was then tested for the chemokine Cxcl-10 by ELISA. The cells transfected with C16 and DNA produced significantly lower levels of Cxcl-10 protein than cells transfected with empty vector.

Example 5

Since Ku forms the heterotrimeric complex DNA-PK with the catalytic subunit (DNA-PKcs), knockout MEFs for Ku70, Ku86 and DNA-PKcs were obtained. The hypothesis that these molecules would be crucial to the innate immune response to DNA was confirmed by showing that these cells are deficient in the production of inflammatory chemokines and cytokines in response to DNA but not to other innate immune stimulants such as poly (I:C) and lipopolysaccharide (LPS). See Fig. 5.

Example 6

DNA-PK associates with cytoplasmic DNA. Immunoblots of biotinylated-DNA/protein complexes precipitated with straptavidin from the cytoplasmic fraction of HEK293T cells at 0, 60 and 120 minutes following transfection. All three components of DNA-PK; Ku70, Ku80/86 and DNA-PKcs, are found bound to cytoplasmic DNA. WCL, whole cell lysate. AP, affinity purification. See fig. 6.

Example 7

To identify novel DNA sensors, biotinylated dsDNA oligonucleotides were transfected into HEK293T cells, cytoplasmic DNA/protein complexes were isolated and the components analysed by SDS-PAGE and mass-spectrometry. Peptide signatures from three distinct proteins that bound dsDNA were identified as Ku70, Ku80/86 and DNA-PKcs. These proteins constitute the DNA-PK heterotrimer, a complex that until now was largely uncharacterised in terms of cytoplasmic function.

A role for DNA-PK in innate immunity was suggested by the upregulation of *Ku70*, *Ku80* and *DNA-PKcs* upon stimulation with DNA (Figure 7). The detection of cytoplasmic dsDNA is likely, as with RNA detection, to be dependent on sequence and structure and accordingly several DNA types were assessed for induction of chemokines when transfected into MEFs (Fig. 5a).

Upregulation of *DNA-PKcs*, *Ku70* and *Ku80* transcription following stimulation with dsDNA. MEFs were stimulated with 10 μg/ml dsDNA for the indicated times and the level of transcription of the indicated genes was measured by qPCR. See Fig. 7.

Example 8

A 45-bp dsDNA was a weak stimulator of *Cxcl10* (IP-10) transcription. However, when this was concatamerised (Fig. 8a), there was a striking induction of *Cxcl10* in a dose-dependent manner (Fig. 8b). This observation was mirrored with intact viral and bacterial genomes (Fig. 5a). This stimulation was sensitive to DNase treatment, specifically required transfection into the cytoplasm and was dependent on DNA length, with longer DNAs giving increased stimulation (Fig. 8).

Concatamerisation of oligonucleotides results in dsDNA species capable of activating chemokine and cytokine transcription in fibroblasts**. a,** Annealed complementary 45-bp oligonucleolides (An) become concatamerised into higher order DNA species (Con) following treatment with polynucleotide kinase and T4 DNA ligase. **b**, Transfection of these dsDNA concatamers into fibroblasts results in activation of *Il6* and *Cxcl10* transcription in a dose-dependent manner. **c.** Stimulation of fibroblasts with dsDNA concatamers requires transfection of intact DNA into the cytoplasm. Pre-treatment of concatamers with DNase abrogates production of *Cxcl10* and *Il6* in response to DNA transfection. **d,** Stimulation of MEFs with dsDNA requires transfection since addition of dsDNA to the culture medium without complexing with transfection reagent fails to induce transcription of *Cxcl10*. **e**, Length dependency of dsDNA stimulation is shown by transfection of oligonucleotides of increasing sizes into MEFs and measurement of *Cxcl10* transcription (Fig. 8).

Example 9

DNA-PKcs was crucial for cytokine and chemokine expression in response to DNA (Fig. 18b, c). This dependence covers the classic innate immune, pro-inflammatory response including IFNβ, IL-6 and chemokines CXCL10, CCL5 (Fig. 17b, c) and CCL4 (Fig. 9a). The transcription of *Cxcl2*, *Ccl3*, *Ifng*, *Ccl2*, *Il4* and *Il10* was either not detected or remained unaffected by DNA-PKcs loss (Fig. 9a). However, wild type and *DNA-PKcs*$^{-/-}$ MEFs responded equally to RNA (Fig. 17b) and lipopolysaccharide (LPS)

(Fig. 17c). Interestingly, this innate immune response to DNA increased over time, unlike the production of chemokine RNA following LPS stimulation, which peaked at two hours before returning to basal levels (Fig. 18c) as reported. These data were recapitulated using primary *Ku70*$^{-/-}$ and transformed *Ku80*$^{-/-}$MEFs (Fig. 17d, e), although IFNβ expression was absent from the transformed *Ku80*$^{-/-}$ cells. These data highlight the importance of Ku and DNA-PKcs in the innate response to dsDNA. Furthermore, the transcription of *Ifnb* in response to DNA was impaired in adult murine fibroblasts lacking both *Ku* genes (Fig. 10b) indicating this response is not limited to embryonic cells. The DNA-PK complex is therefore necessary for initiating the innate immune response to cytoplasmic DNA.

Inflammatory cytokine expression in response to DNA is dependent on DNA-PKcs and Ku expression. a, DNA transfection leads to the transcription of cytokines that increases over time and the expression of *Ccl5*, *Il6* and *Ccl4* is dependent on DNA-PKcs whereas *Cxcl2* expression in unaffected by the loss of this gene. No induction of *Ccl2, Ccl3, Il4, Il10* or *Ifng* was found following DNA stimulation. b, In adult murine fibroblasts the transcription of *Ifnb* in response to DNA, but not RNA, is perturbed in the absence of the Ku70 and Ku80. qPCR analysis of RNA harvested from wild type or Ku knockout cells 6 h after transfection with DNA (10 μg/ml) or Poly(I:C) (10 μg/ml). See Fig. 10.

Example 10

The innate immune response to DNA is (**a**) dependent on STING but (**b**) independent of toll-like receptor signalling, IPS-1, DAI, IRF5 and IRF7. qPCR analysis of RNA harvested from MEFs of indicated genotypes 6 h after transfection with DNA (10 μg/ml) or Poly(I:C) (10 μg/ml) or infection with MVA (m.o.i. of 5).

The mechanism by which DNA-PK signals was addressed and showed the response to DNA was dependent on IRF3 (Fig. 14a), TBK1 (Fig. 14b) and STING (Fig. 10a) but independent of TLRs, IPS-1 (and hence RNA-Pol III), DAI, IRF5 and IRF7 since it was equivalent in wild type, *Myd88*$^{-/-}$/Trif$^{-/-}$, *Ips-1*$^{-/-}$, *Dai*$^{-/-}$, *Irf5*$^{-/-}$, and *Irf7*$^{-/-}$MEFs (Fig. 10b). IRF3-dependent signalling is key to this innate immune response, as described. See Fig. 10.

Example 11

The importance of IRF3 in the innate immune response is well documented and DNA-PK can directly activate this transcription factor, consistent with the IRF3 dependence shown in this and previous studies. There is, therefore, a direct and potent activation of the innate immune response through this signalling pathway. The loss of DNA-PK components, Ku or DNA-PKcs, results in a clear and significant reduction in cytokine and chemokine mRNA production in response to DNA transfection and, importantly, VACV infection.

The relevance of this novel DNA sensing mechanism to virus infection was tested using MVA, an attenuated VACV strain that lacks many inhibitors of immune signalling and hence is a good model for viral immune stimulation. MVA activates innate immunity via TLR-dependent and independent pathways and during MVA infection viral DNA accumulates in cytoplasmic virus factories. IRF3 was not found localised with factories in infected cells, but in the nucleus, reflecting its activation by virus infection (Fig. 11a).

The synthesis of MVA proteins and DNA in MEFs was unaffected by the loss of *Ku80* (Fig. 12b and c), probably due to MVA inhibition of IFNβ expression.

a IRF3 does not localise to MVA DNA factories following infection in HeLa cells. Scale bar; 5 µm. **b** MVA replication, and **c** MVA protein synthesis, in MEFs is unaffected by loss of Ku80. See Fig. 11.

Example 12

The absence of DNA-PKcs had little effect on *Cxcl10* transcription in response to HSV-1 infection (Fig. 12) reflecting the lack of exposure of the HSV-1 genome to the cytoplasmic compartment.

The innate immune response to HSV-1 infection is not dependent on DNA-PKcs. MEFs were infected with HSV-1 (m.o.i of 1) for 6 h and the transcription of *Cxcl10* was analysed by qPCR. See Fig. 12.

Example 13

DNA-PK components are expressed in a wide range of tissues and cell types although are absent in primary macrophages (Fig. 13). In contrast, AIM2 expression is restricted to myeloid cells indicating that there are distinct DNA sensing pathways operating in different cell types.

DNA-PK is expressed in fibroblasts but not primary macrophages. Immunoblotting of protein lysates (50 µg) from murine cells indicating the differential expression of DNA-PK components. See Fig. 13.

Example 14

DNA-PK interacts with IRF3 and activates an innate immune response. a, Transcription of *Cxcl10* and *Ifnb*, analysed by qPCR 6 h after DNA transfection, was abrogated in MEFs lacking *Irf3* when compared to wild type cells. b, The expression of *Cxcl10* in response to DNA transfection is dependent on TBK-1. c, IRF3 nuclear translocation is inhibited in *DNA-PKcs-/-* cells in response to DNA but not RNA, quantified in d (n=3, counts of at least 50 nuclei per slide). e, The stimulation of *Isg54* by dsDNA is dependent on IRF3 (left panel) and DNA-PKcs (right panel). *Isg54* transcription was measured by qPCR 6 h following DNA (10 µg/ml) or IFNβ (2000 U/ml) stimulation. f, Coimmunoprecipitation of IRF3 with DNA-PKcs and Ku80. Expression of IRF3 in HeLa cells followed by immunoprecipitation using either anti-IRF3 (left panel) or anti-Ku80 (right panel) and immunoblotting for indicated proteins. –ve: control IgG. ** $p<0.01$, * $p<0.05$, n=3, error bars +/- SEM. ns; non-stimulated. Scale bar; 5 µm. See Fig. 14.

In *DNA-PKcs*$^{-/-}$MEFs the nuclear translocation of IRF3 was abrogated in response to DNA, but not RNA, transfection (Fig. 15c and d), indicating that DNA-PK acts upstream of IRF3. Accordingly, IRF3 activity following DNA stimulation was measured by the transcriptional activation of the IFN-inducible gene *Isg54* and was entirely dependent on the presence of both IRF3 and DNA-PKcs (Fig. 15e). In contrast, *Isg54* activation by IFNβ was unperturbed in *DNA-PKcs*$^{-/-}$ MEFs. DNA-PK can interact directly with IRF3 and contribute to its activation *in vivo* and this interaction was reproduced here (Fig. 15f). The DNA sensing pathway is therefore dependent on DNA-PK activating cytokine production in an IRF3-dependent manner.

DNA-PKcs$^{-/-}$and *Ku80*$^{-/-}$MEFs showed a significant reduction in cytokine and chemokine mRNA production in response to MVA infection (Fig. 15b, c) and this response was IRF3-dependent (Fig. 14a). This is direct evidence that the innate immune response to viral infection is regulated by DNA-PK and confirms its role in cytoplasmic DNA sensing.

Example 15

DNA-PK is important for the innate immune response to VACV (strain MVA) infection. **a**, Ku70 and DNA-PKcs co-localise with VACV DNA factories by immunofluoresence. Cytoplasmic viral factories formed after MVA infection of HeLa cells and visualised with DAPI (red) co-localise with both Ku70

(left panel, green) and DNA-PKcs (right panel, green). Sites of co-localisation are indicated by white arrows and by the overlaid images where yellow indicates co-staining, Scale bar; 5 μM. **b** and **c**, In *DNA-PKcs-/-* or *Ku80-/-* cells, cytokine transcription is impaired following MVA infection. qPCR analysis of *Cxcl10* and *Il6* transcription in *DNA-PKcs-/-* and *Ku80-/-* MEFs following infection with increasing doses of MVA (left panels, representative of three independent 14 experiments) or 6 h after infection, m.o.i.=5 (right panels). ** $p<0.01$, n=3, error bars +/- SEM, ni; non-infected. See Fig. 15.

Notably, DNA-PK components accumulated in these factories (Fig. 16a), consistent with i) their role in sensing cytoplasmic DNA and ii) the affinity of DNA-PK for DNA hairpins that are present at poxvirus genome termini.

The relative defect in chemokine production after MVA infection was more severe in *DNA-PKcs*$^{-/-}$ than *Ku80*$^{-/-}$cells (Fig. 16a, c) and this is consistent with previous reports that DNA-PKcs can bind DNA in the absence of Ku, but with a lower affinity.

Example 16

Figure 16 shows that deleting the C-terminal region of C16 does not delete inhibitory function/blocking activity but deleting the N-terminal does. The minimum portion identified as functional is: MDIYDDKGLQTIKLFNNEFDCIRNDIRELFKHVTDSDSIQLPMEDNSDIIE NIRKILYRRLKNVECVDIDSTITFMKYDPNDDNKRTCSNWVPLTNNYMEYCLVIYLETPICG GKIKLYHPTGNIKSDKDIMFAKTLDFKSKKVLTGRKTIAVLDISVSYNRSMTTIHYNDDVDI DIHTDKNGKELCYCYITIDDHYLVDVET

Example 17

DNA-PK is critical for the innate immune response to DNA. **a**, Various dsDNAs stimulate *Cxcl10* transcription when transfected into fibroblasts, as analysed by qPCR. **b**, In *DNA-PKcs-/-* cells, IFNβ and CXCL10 expression and *Ccl5* and *Il6* transcription are abrogated in response to DNA, but not RNA transfection. Responses measured by ELISA or qPCR 6h after transfection. **c**, *Cxcl10* transcription increases over time in following DNA stimulation, but is absent in *DNA-PKcs-/-* MEFs (left panel). There is an unimpaired response to LPS treatment in these cells (right panel). **d**, In *Ku80-/-* cells the induction of *Cxcl10* and *Il6* is significantly impaired following DNA stimulation. *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n=3, error bars +/- SEM, ns; nonstimulated. Data shown are representative of at least two separate experiments. See Fig. 18.

Example 18a

DNA-PKcs kinase activity is not required for DNA sensing. qPCR detection of CXCL10 transcription following stimulation of mouse embryonic fibroblasts from wild type (Balb/c) or DNA-PKcs kinase-mutant (SCID) mice with DNA.

Example 19

DNA-PKcs kinase activity is not required for DNA sensing *in vivo*. qPCR detection of CXCL10 transcription 8 hours following injection of wild type (Balb/c) or DNA-PKcs kinase-mutant (SCID) mice with DNA, RNA or MVA. Lipofectamine 2000 (LF2K) injection was used as a negative control.

Example 20

Minimum number of copies of GAGA identified per virus strain

Virus Strain	Number of Copies of GAGA motif
Vaccinia-WR	26
Vaccinia-MVA	134
Horsepox MNR76	2
Variola- Garcia	1
Variola- Bangladesh	1
Camelpox- CMS	2
Cowpox- Brighton Red	68
Cowpox- Germany 91-3	10
Cowpox- GRI_90	6
Adenovirus- B1	2
Adenovirus-B2	2
Adenovirus-D	1
Adenovirus-type 35	2
Adenovirus-type 7	1
Adenovirus-type 11	2

Example 21

C16 is a 37.5 kDa protein, which is expressed early during infection and localizes to the cell nucleus and cytoplasm of infected and transfected cells. The loss of the *C16L* gene had no effect on virus growth kinetics but did reduce plaque size slightly. The *C16L* gene is present in the inverted terminal repeat and so is one of the few VACV genes that are diploid. The C16 protein is highly conserved between different VACV strains, and also in the orthopoxviruses variola virus, ectromelia virus, horsepox virus and cowpox virus.

Furthermore, the virulence of a virus lacking *C16L* (vΔC16) was reduced in a murine intranasal model compared with control viruses (Fahy et al., J Gen Virol, 2008) and there were reduced virus titres from 4 days post-infection.

In the absence of C16, the recruitment of inflammatory cells in the lung and bronchoalveolar lavage was increased early after infection (day 3) and more CD4+ and CD8+ T cells expressed the CD69 activation marker. Conversely, late after infection with vΔC16 (day 10) there were fewer T cells remaining, indicating more rapid clearance of infection. Collectively, these data indicate that C16 diminishes the immune response and is an intracellular immunomodulator.

Example 22

Virus lacking C16 induces greater specific inflammatory mediators

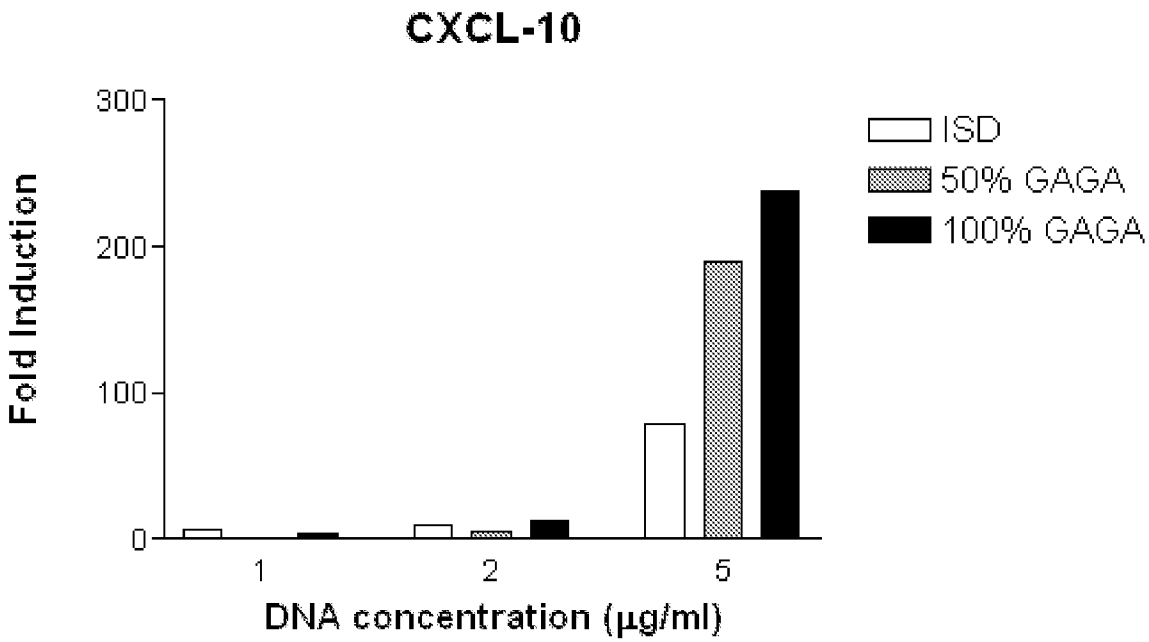
Groups of 5 BALB/c mice were infected wither either WR, knockout C16 virus (vΔC16) or revertant virus. A) BAL samples were taken at the specified timepoints and CXCL10 concentrations measured by ELISA. B.) BAL samples were taken at the specified timepoints and IL-6 protein concentrations were measured at the specified timepoints by ELISA. C) Tracheas from infected mice were harvested and IFNβ mRNA levels were assessed by qRT-PCR.

Claims

1. An isolated polynucleotide sequence comprising a region rich in the motif GAGA for example rich in the motif $([X]_{0-50}GAGAAAGAGAA[Y]_{0-50})n$ wherein n is an integer 1 to 150, X or each X is independently selected from a base and Y or each Y is independently selected from a base.
2. An isolated polynucleotide sequence according to claim 1 for generating an innate immune response *in vivo*.
3. An isolated polynucleotide sequence according to any one of claims 1 or 2, wherein the sequence comprises in the range 10 to 250, for example 45 and 200 base pairs, such as 50 to 100 base pairs in length.
4. An isolated polynucleotide sequence according to any one of claims 1 to 3, wherein the sequence is single stranded.
5. An isolated polynucleotide sequence according to any one of claims 1 to 3, wherein the sequence is double stranded.
6. An isolated polynucleotide sequence according to any one of claims 1 to 5, wherein the polynucleotide is DNA.
7. An isolated polynucleotide sequence according to any one of claims 1 to 6, wherein the sequence consists of or consists essentially of GAGA repeats.
8. A composition comprising a polynucleotide according to any one of claims 1 to 7.
9. A pharmaceutical composition comprising a polynucleotide according to any one of claims 1 to 7.
10. A pharmaceutical composition according to claim 9, wherein the composition is a vaccine composition and further comprises a biologically active agent such as an antigen.
11. A pharmaceutical composition according to claim 10 wherein the vaccine composition is a therapeutic vaccine composition.
12. A pharmaceutical composition according to claim 10 wherein the vaccine composition is a prophylactic vaccine composition.
13. A polynucleotide as defined in any one of claims 1 to 7 or a composition as defined in claim 8 to 12, for use in treatment.
14. A polynucleotide as defined in any one of claims 1 to 7 or a composition as defined in claim 8 to 12, for use in generating or boosting an immune response.
15. A polynucleotide or composition according to claim 14, for use in generating or boosting an innate immune response.
16. A polynucleotide as defined in any one of claims 1 to 7 or a composition as defined in claim 8 to 12, for use as an adjuvant.
17. A polynucleotide or composition according to claim 16, wherein a TH1 and/or TH2 immune response is generated or bolstered.
18. A polynucleotide or composition according to any one of claims 1 to 17, for use as an antiviral agent.
19. A method of generating or boosting an innate immune response comprising administering a therapeutically effective amount of a polynucleotide according to any one of claims 1 to 7 or a composition according to any one of claims 8 to 12.

20. A method of modulating immunogenicity of a polynucleotide by inserting or deleting a sequence comprising 'GAGA' polynucleotide.
21. A method of genetically engineering a virus with an altered GAGA copy number by increasing or decreasing the numbers of 'GAGA' therein.
22. A method according to claim 21 wherein the GAGA copy number is increased.
23. A method according to claim 21 wherein the GAGA copy number is decreased, for example to zero.
24. A method according to any one of claims 21 to 23, wherein the virus has a gene encoding C16 and/or genes encoding similar proteins which inhibit DNA-PK mediated DNA sensing, silenced or deleted .
25. A virus obtained or obtainable by the method of any one of claims 21 to 24.
26. A virus of claim 25 for use in therapy, for example immunoprophylaxis and/or immunotherapy.
27. A virus of claim 25 wherein said virus has a low or zero copy number for use in gene therapy.
28. A virus of claim 25 for use as a vector.
29. Isolated DNA-PK for use as a DNA sensor.
30. A method of using DNA-PK as a DNA sensor comprising the step of detecting foreign DNA.
31. A method of stimulating an innate immune response comprising the step of detecting foreign DNA using DNA-PK.
32. A chemical entity, polypeptide or protein which inhibits stimulation of innate immunity mediated via DNA-PK.
33. A chemical entity, polypeptide or protein which interacts with the Ku complex to inhibit stimulation of innate immunity mediated via DNA-PK.
34. A polypeptide or protein according to any one of claims 32 or 33 wherein the polypeptide or protein is C16 or an active fragment thereof.
35. C16 or an active fragment thereof for use in therapy, in particular for inhibiting stimulation of innate immune response.
36. C16 or an active fragment thereof according to claim 35, wherein said innate immune response is mediated by DNA-PK.
37. C16 for use as an inhibitor of DNA-PK.
38. A method of screening for modulators of innate immunity comprising the step:
admixing the potential modulator with and active form of DNA-PK or components comprising the same, optionally in the presence of a control; and
measuring the levels of one or more inflammatory cytokines, chemokines or interferons, for example selected from CXCL-10, IL-6, CCL-5, IFN-b.
39. *Ex vivo* or *in vitro* use of an isolated polynucleotide sequence comprising a region rich in the motif GAGA for example rich in the motif $([X]_{0-50}GAGAAAGAGAA[Y]_{0-50})_n$ wherein n is an integer 1 to 100, X is independently selected from a base and Y is independently selected from a base, to generate an innate immune response.

FIGURE 1



Titration of DNA into cells and measurement of CXCL-10 induction.

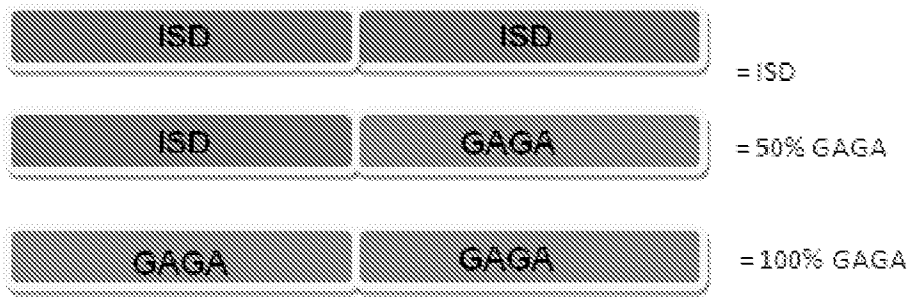


FIGURE 2

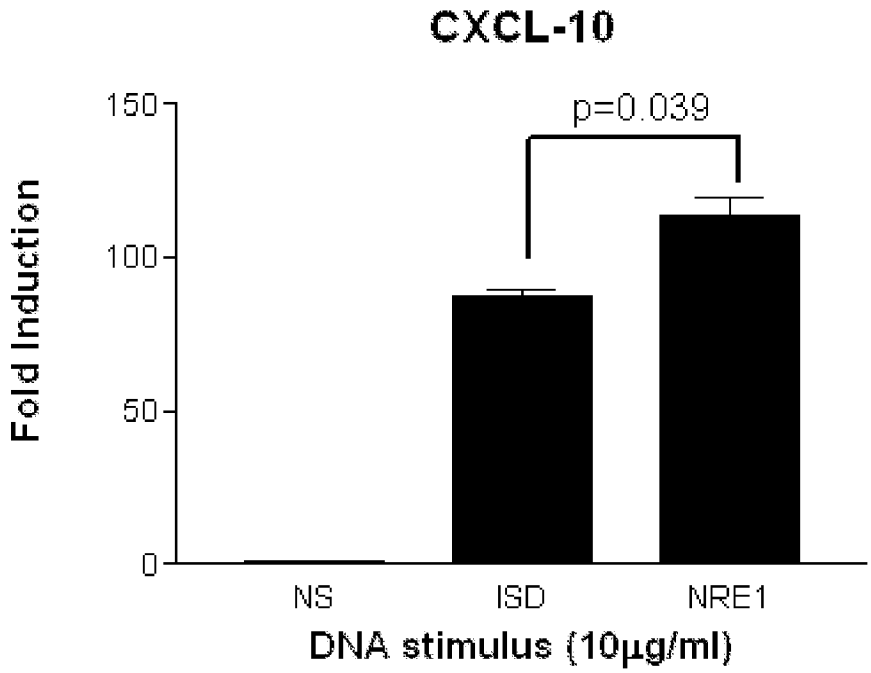


Figure 2. The DNA target sequence for Ku (NRE1) is immunostimulatory.

Figure 3a

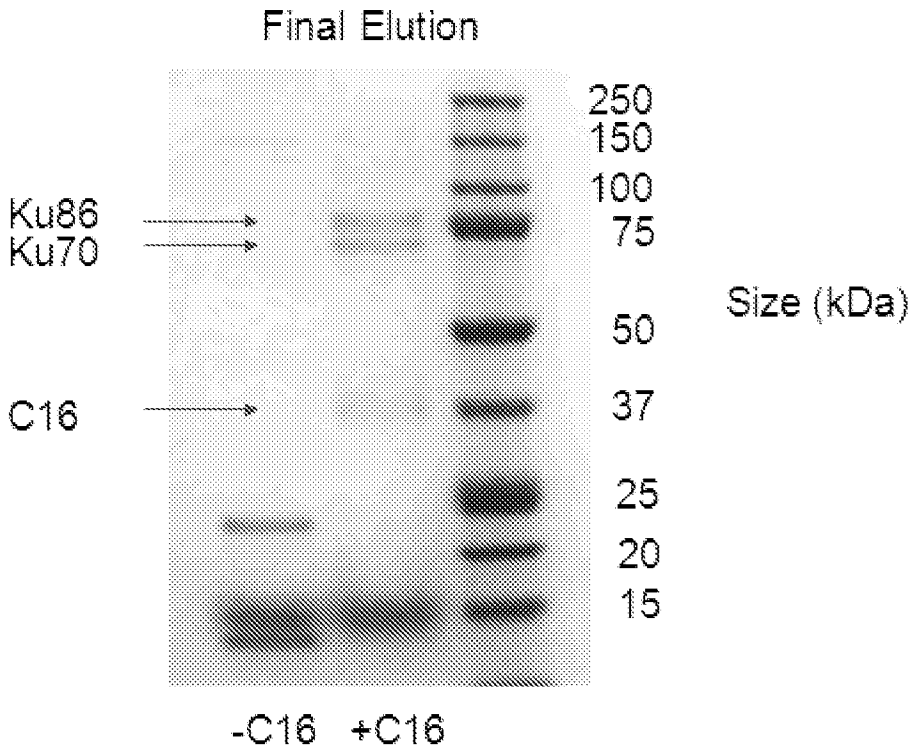


Figure 3b

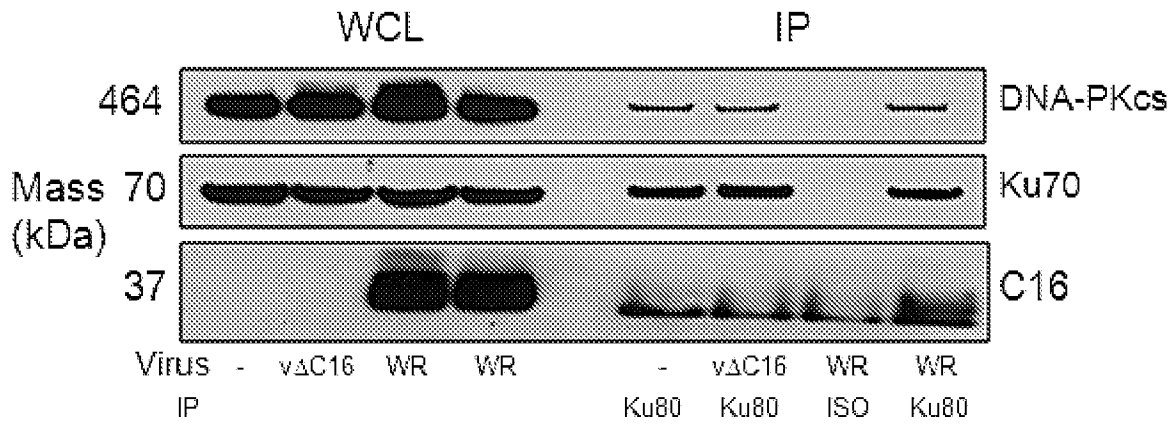
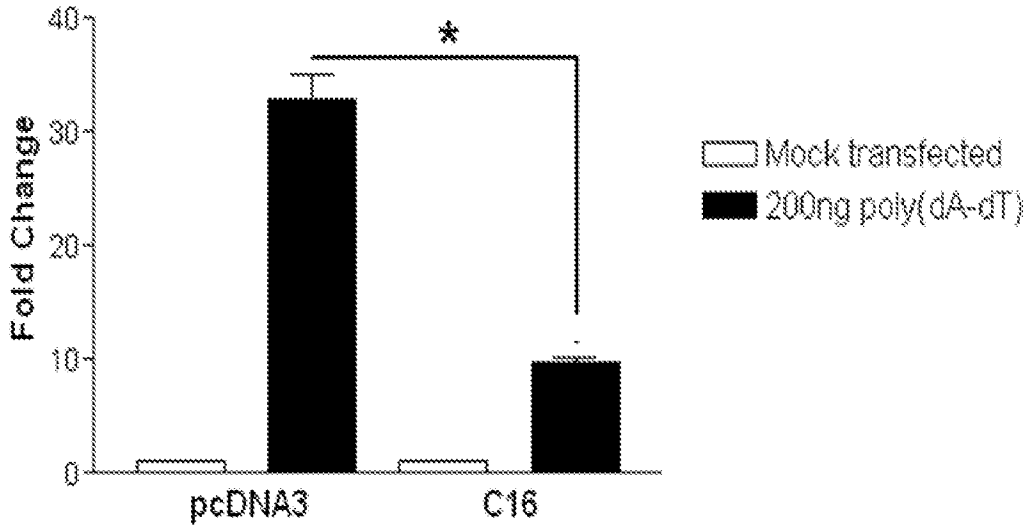


Figure 4


40
30
20
10
0
Fold Change
*
pcDNA3
C16
Mock transfected
200ng poly(dA-dT)

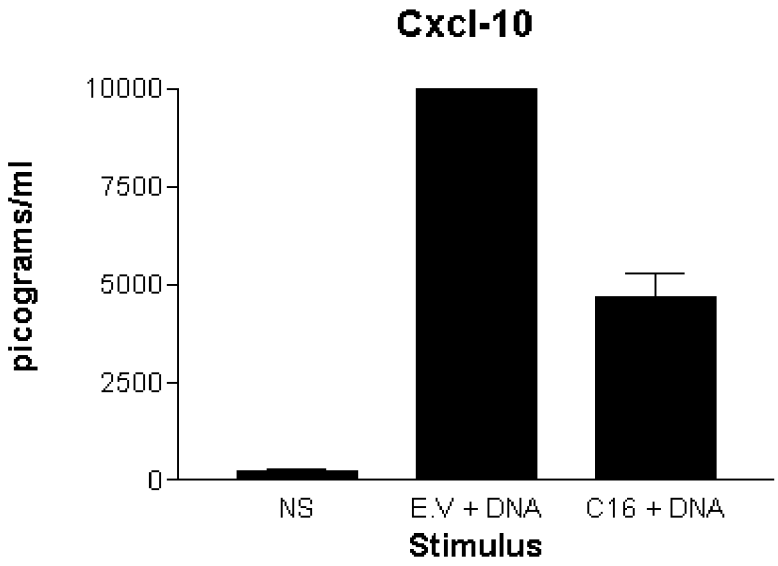

Cxcl-10
10000
7500
5000
2500
0
picograms/ml
NS
E.V + DNA
C16 + DNA
Stimulus

Figure 5

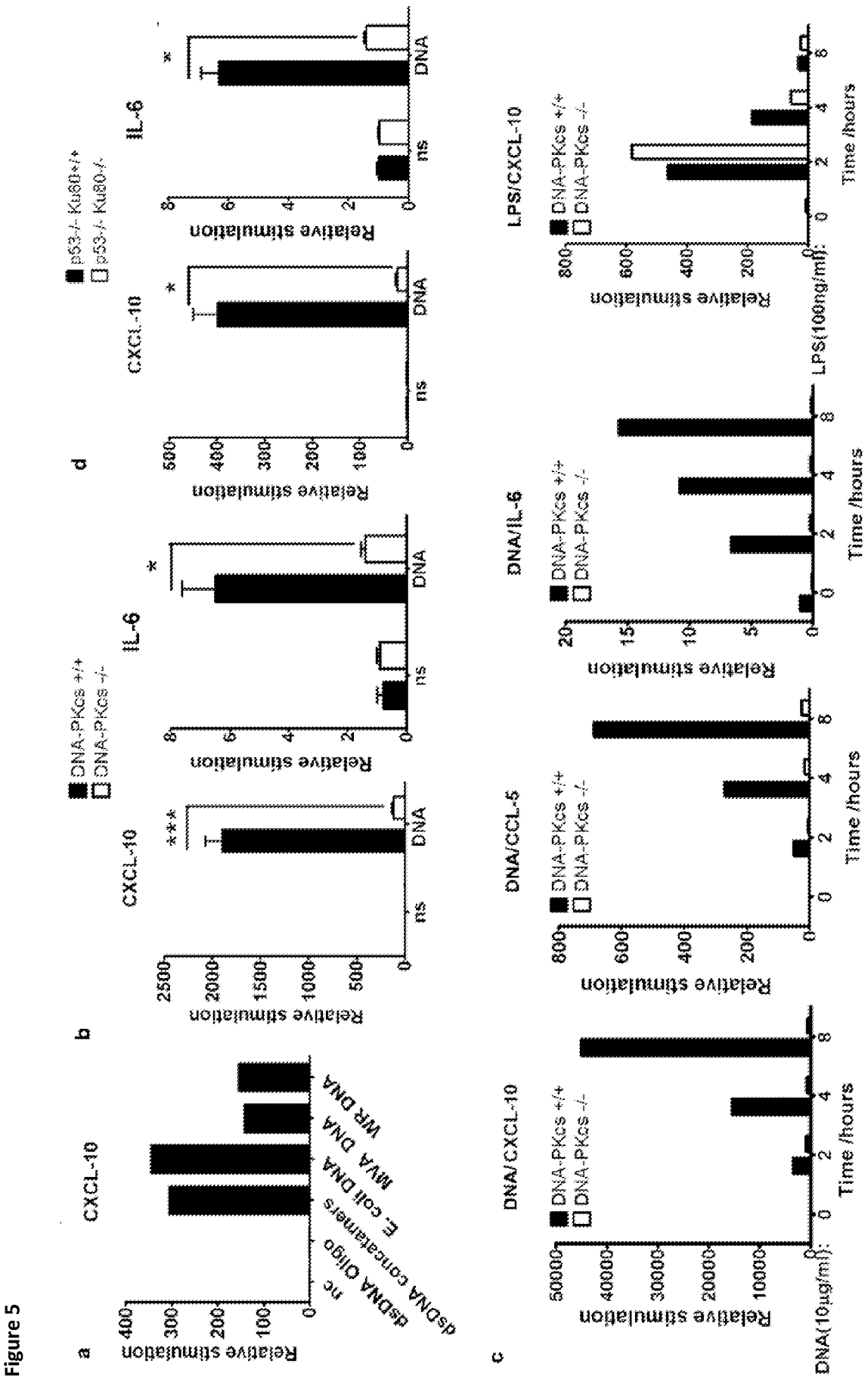

a
CXCL-10
Relative stimulation
nc
dsDNA Oligo
dsDNA concatamers
E. coli DNA
MVA DNA
WR DNA
b
CXCL-10
IL-6
DNA-PKcs +/+
DNA-PKcs -/-
***
*
ns
DNA
c
DNA/CXCL-10
DNA/CCL-5
DNA/IL-6
LPS/CXCL-10
DNA(10µg/ml):
LPS(100ng/ml):
Time /hours
d
p53-/- Ku80+/+
p53-/- Ku80-/-

Figure 6

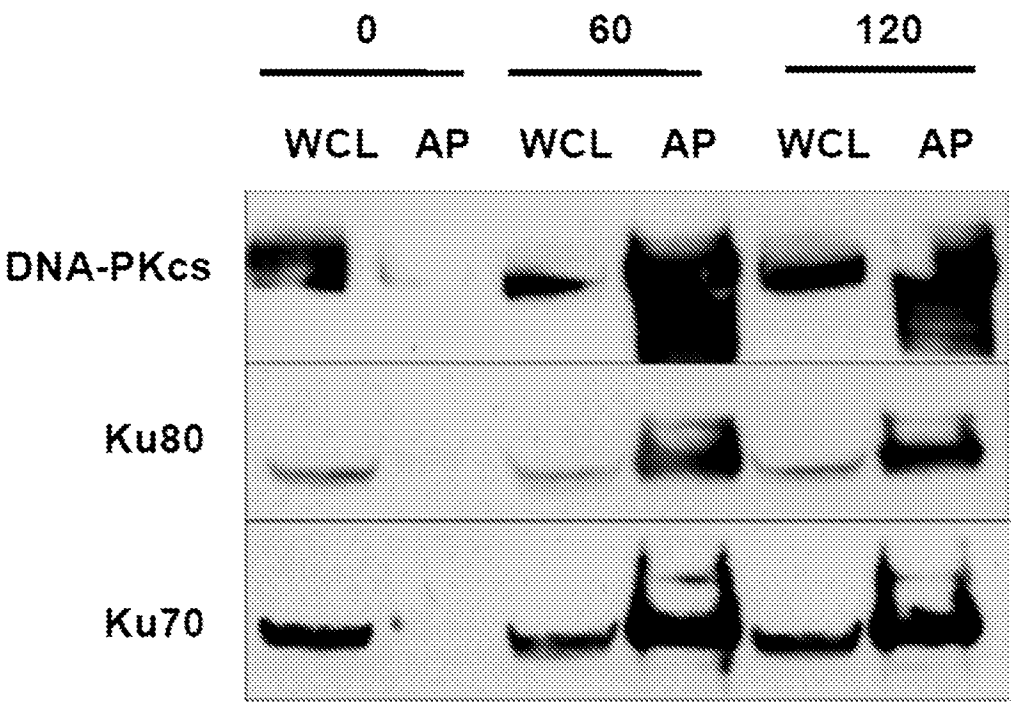


Figure 7

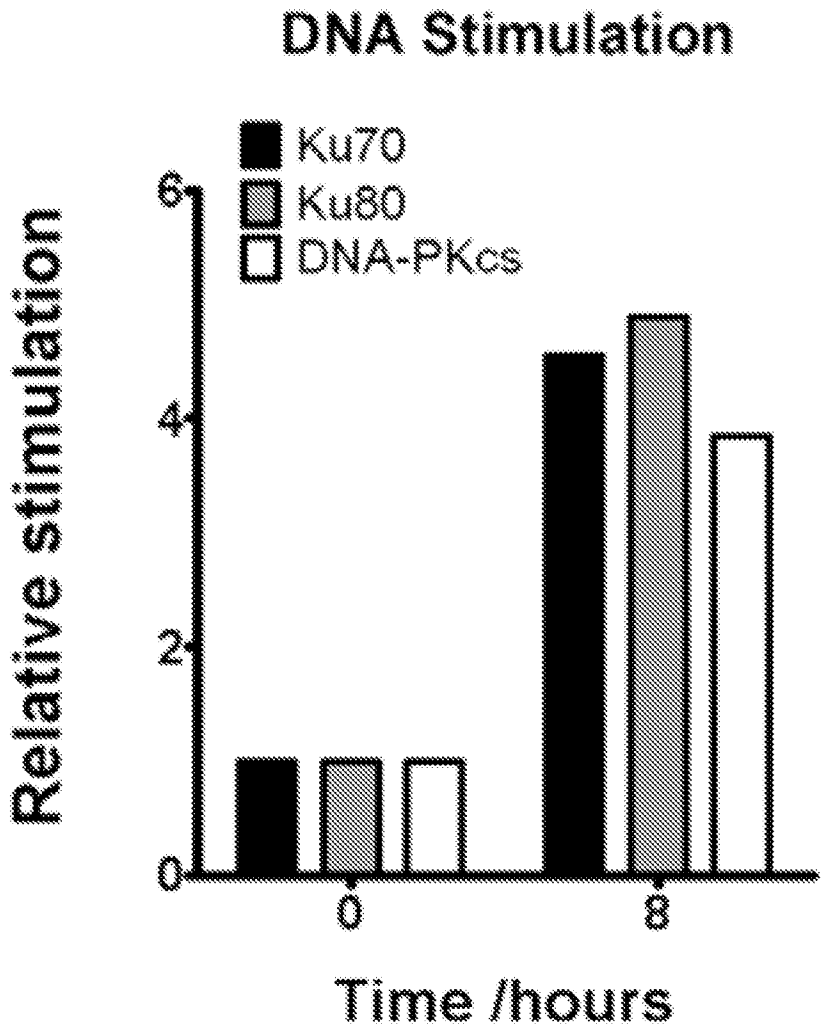

DNA Stimulation
Ku70
Ku80
DNA-PKcs
Relative stimulation
6
4
2
0
0
8
Time /hours

Figure 8

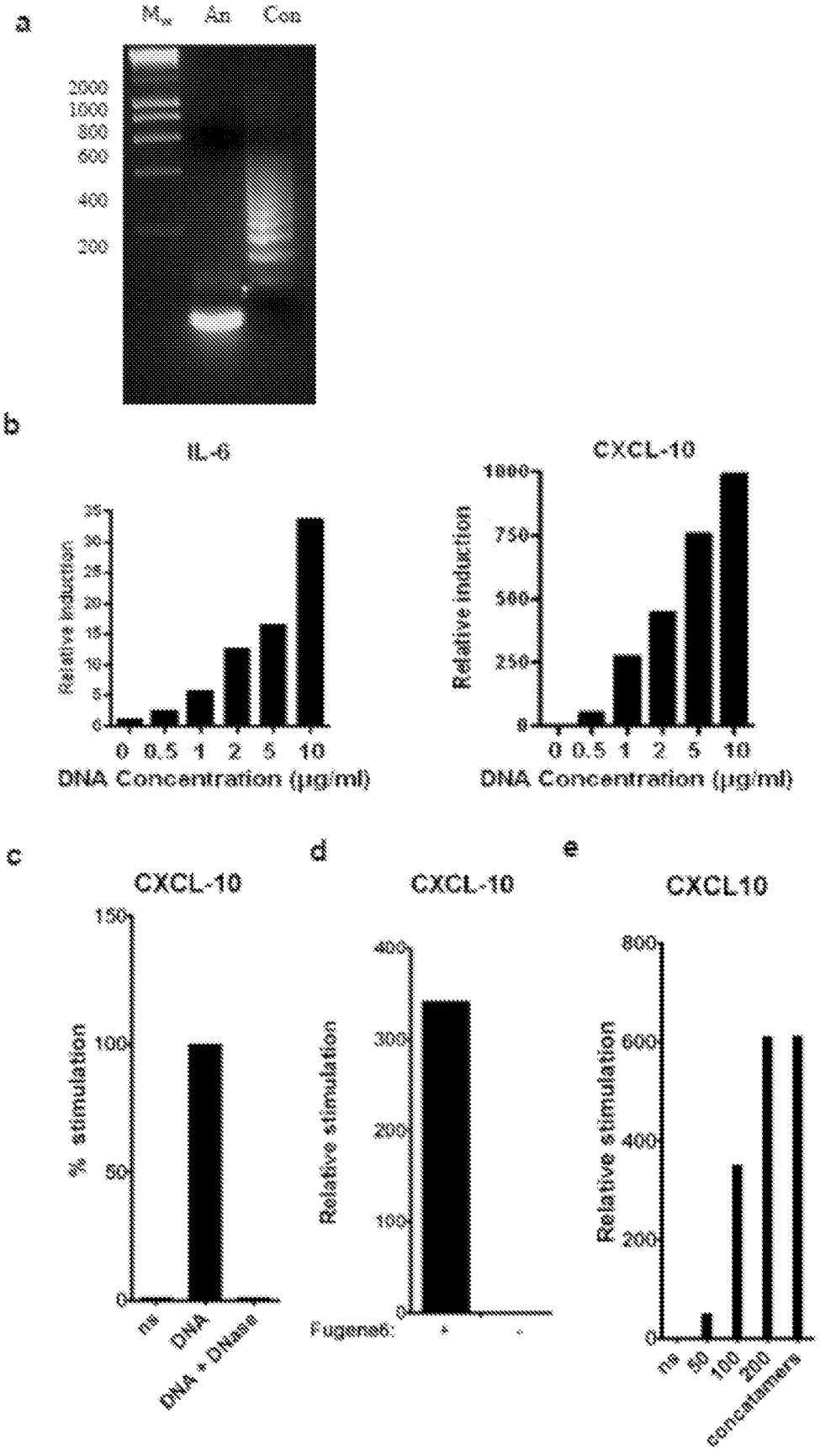

a
Mw
An
Con
2000
1000
800
600
400
200
b
IL-6
Relative induction
0 0.5 1 2 5 10
DNA Concentration (µg/ml)
CXCL-10
Relative induction
1000
750
500
250
0
0 0.5 1 2 5 10
DNA Concentration (µg/ml)
c
CXCL-10
% stimulation
150
100
50
0
ns
DNA
DNA + DNase
d
CXCL-10
Relative stimulation
400
300
200
100
0
Fugene6:
+
-
e
CXCL10
Relative stimulation
800
600
400
200
0
ns
50
100
200
concentrations

Figure 9

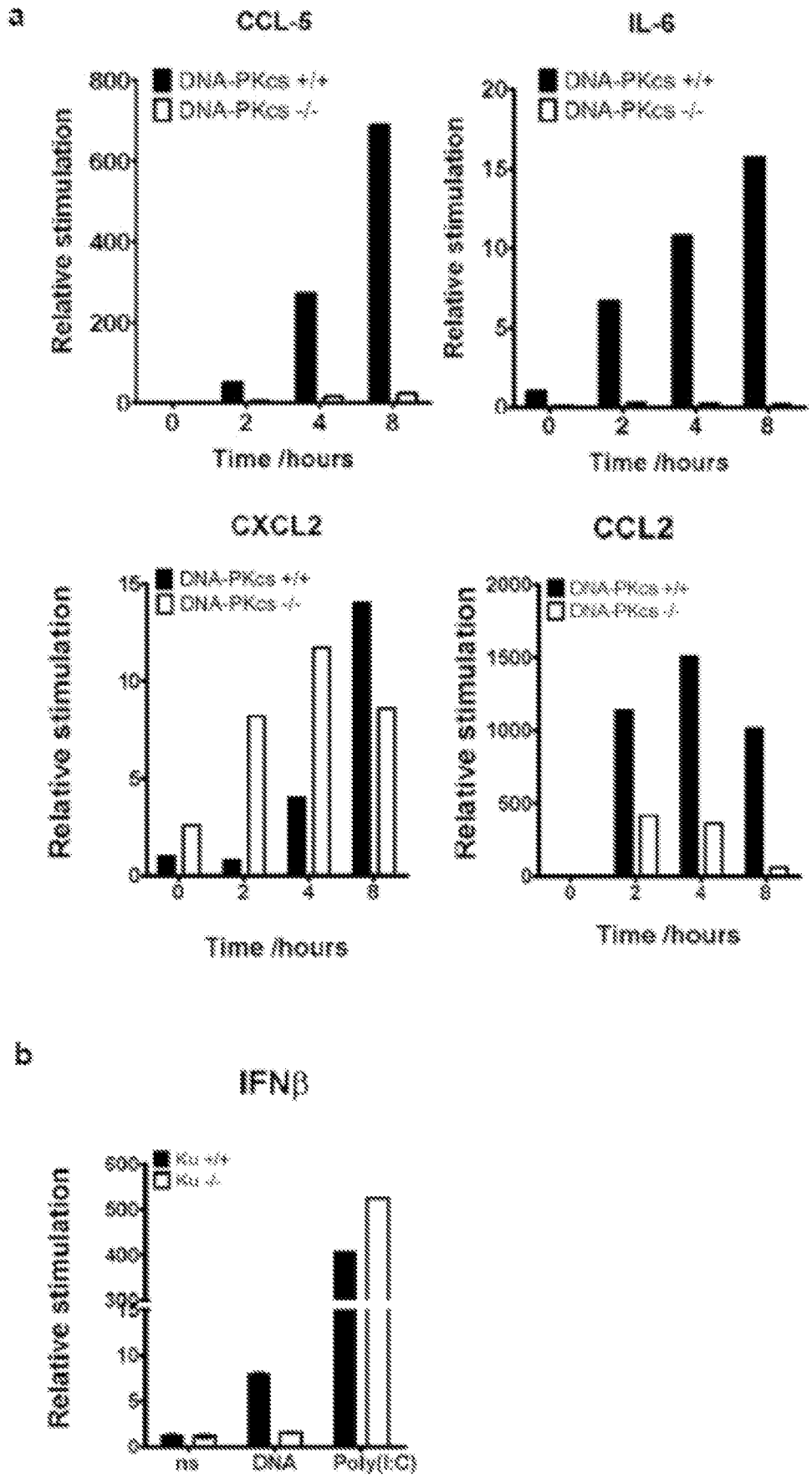

a
CCL-6
IL-6
CXCL2
CCL2
DNA-PKcs +/+
DNA-PKcs -/-
Relative stimulation
Time /hours
0
2
4
8
b
IFNβ
Ku +/+
Ku -/-
ns
DNA
Poly(I:C)

Figure 10

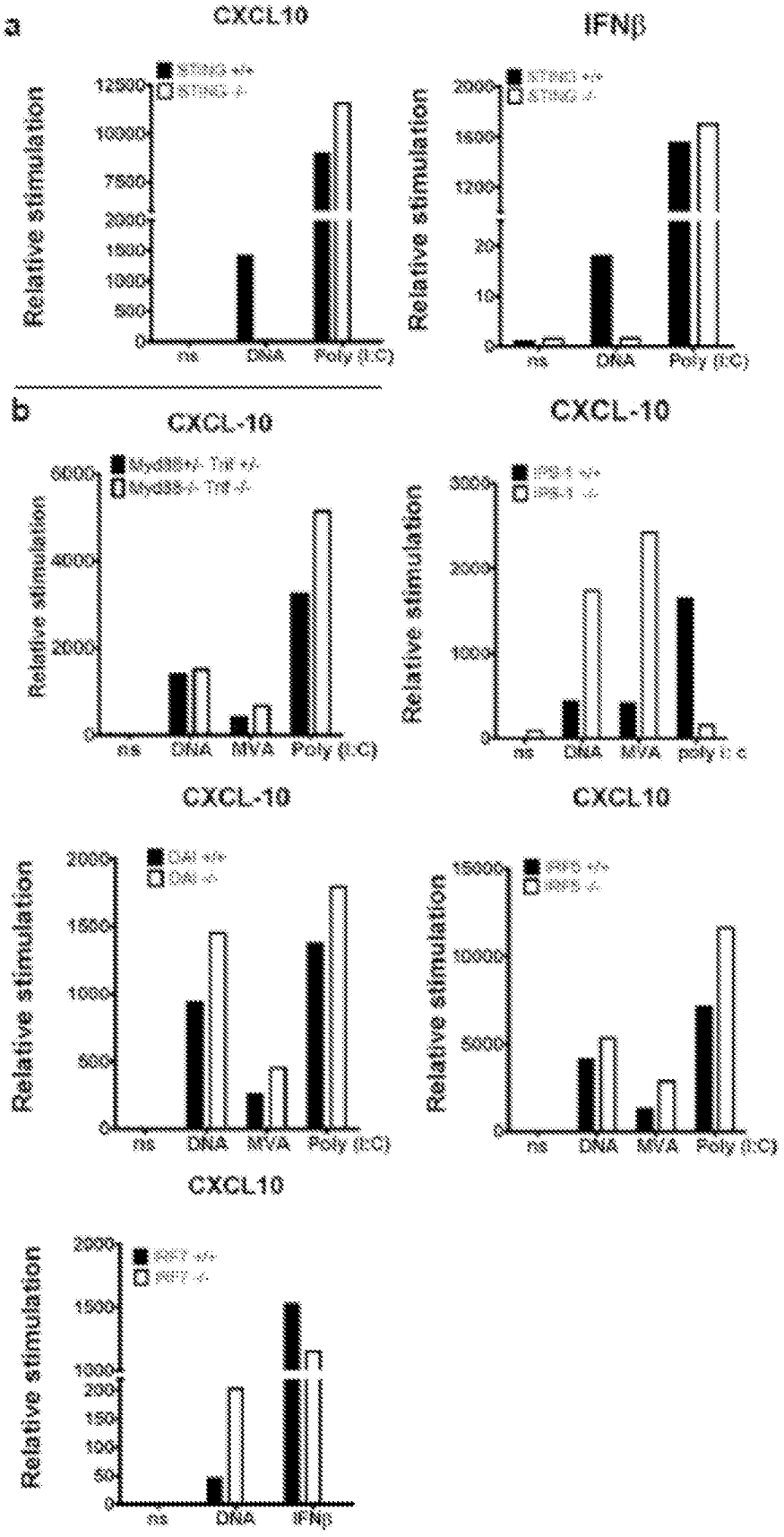
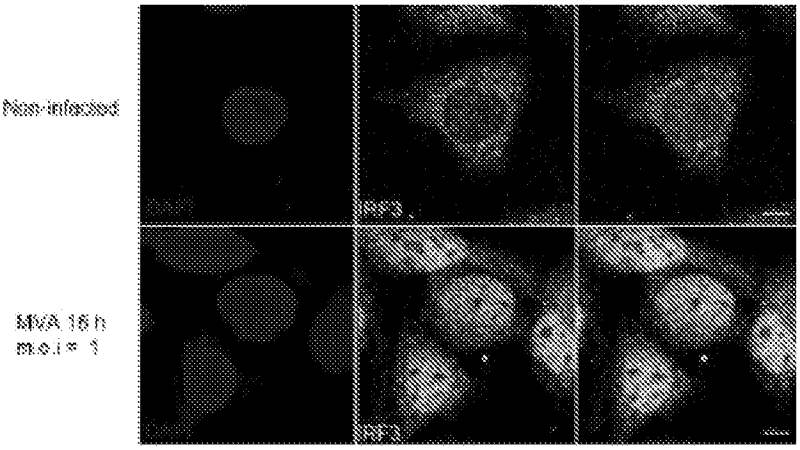
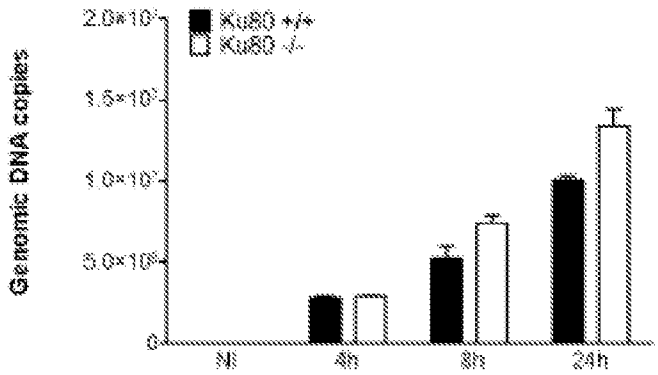

a
CXCL10
IFNβ
Relative stimulation
ns
DNA
Poly (I:C)
b
CXCL-10
CXCL-10
Relative stimulation
ns
DNA
MVA
Poly (I:C)
CXCL-10
CXCL10
CXCL10
IFNβ

Figure 11

a



b



c

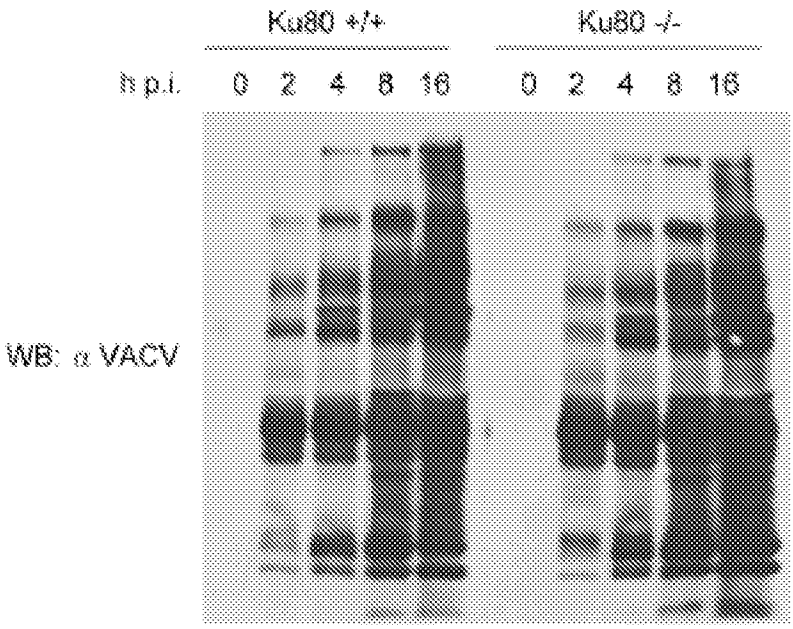


Figure 12

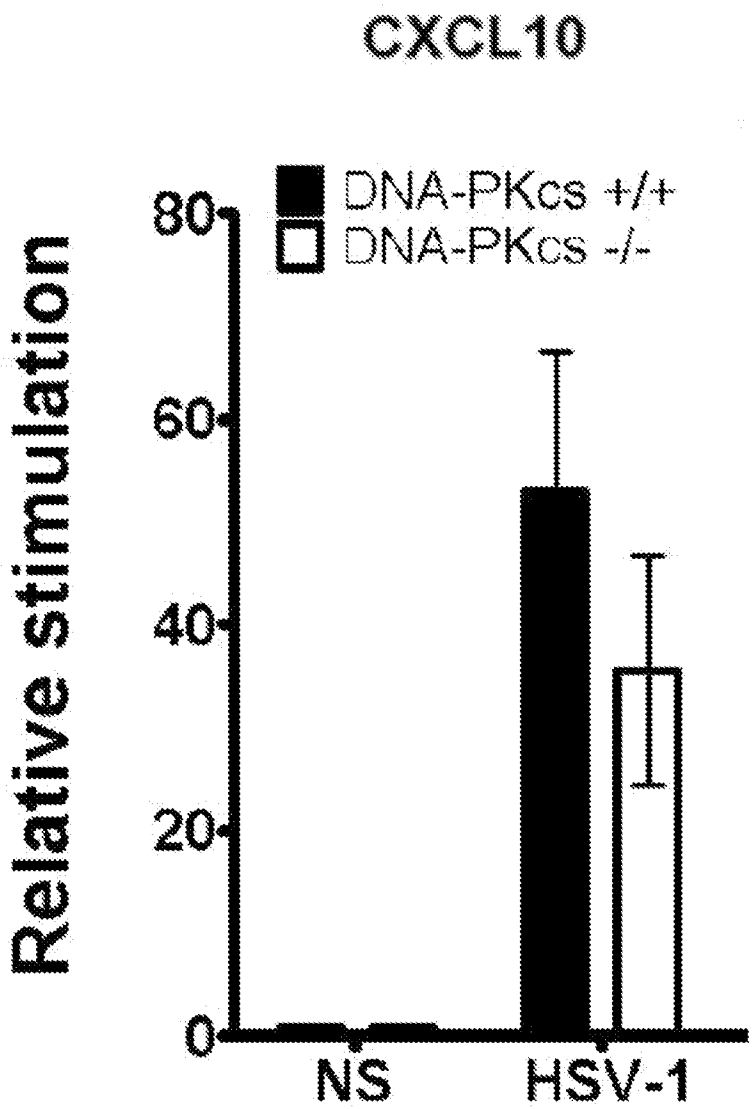

CXCL10
DNA-PKcs +/+
DNA-PKcs -/-
Relative stimulation
80
60
40
20
0
NS
HSV-1

Figure 13

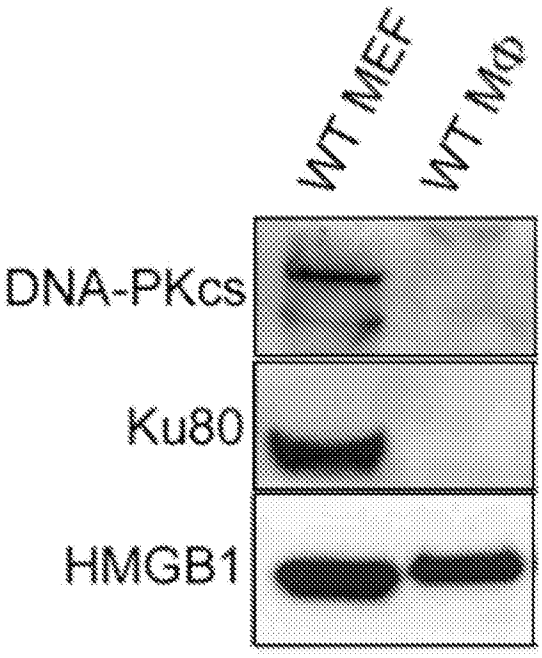


Figure 14

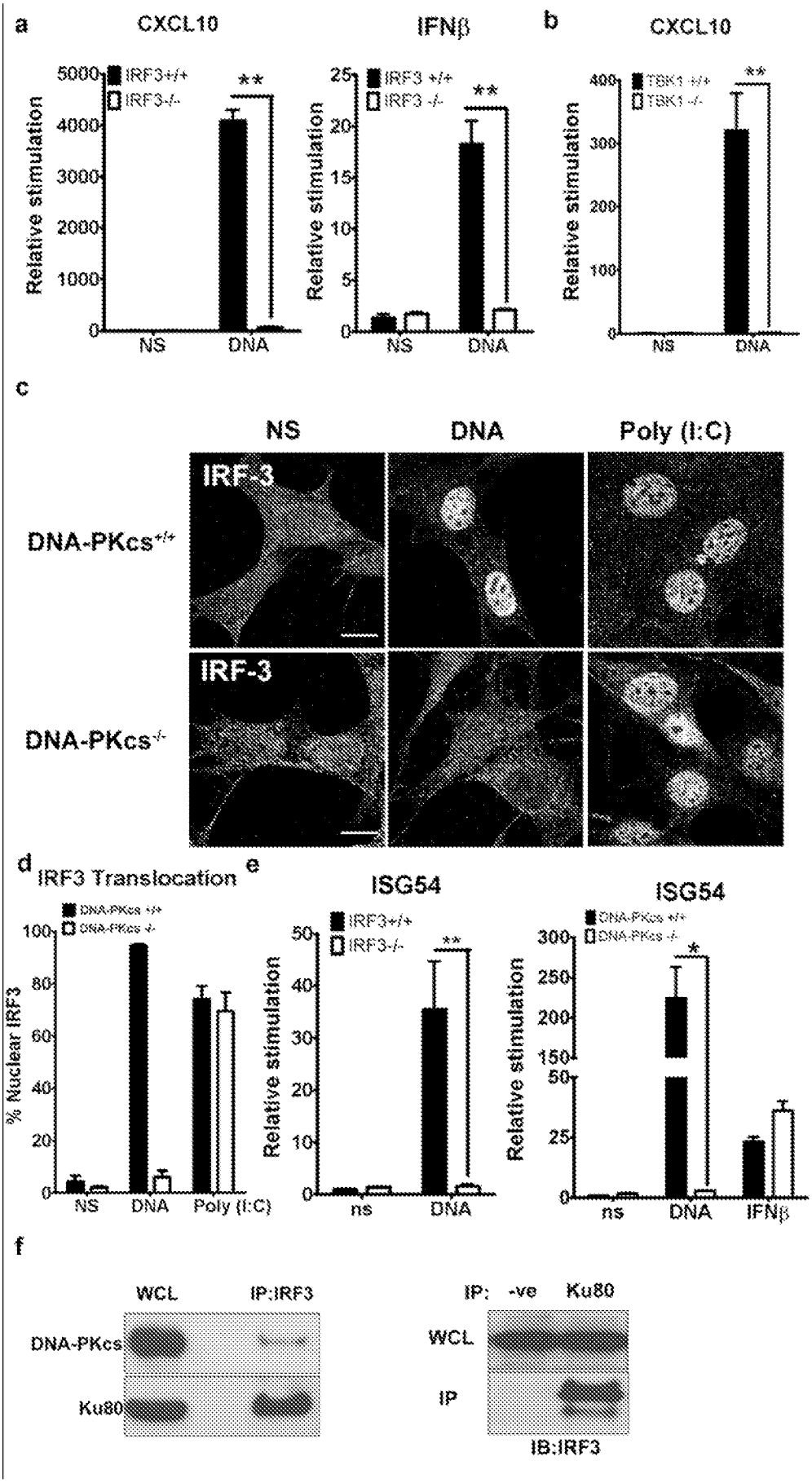

a
CXCL10
IFNβ
b
CXCL10
IRF3+/+
IRF3-/-
IRF3 +/+
IRF3 -/-
TBK1 +/+
TBK1 -/-
Relative stimulation
NS
DNA
c
NS
DNA
Poly (I:C)
IRF-3
DNA-PKcs+/+
DNA-PKcs-/-
d
IRF3 Translocation
DNA-PKcs +/+
DNA-PKcs -/-
% Nuclear IRF3
Poly (I:C)
e
ISG54
ns
IFNβ
f
WCL
IP:IRF3
DNA-PKcs
Ku80
IP: -ve
Ku80
IP
IB:IRF3

Figure 15

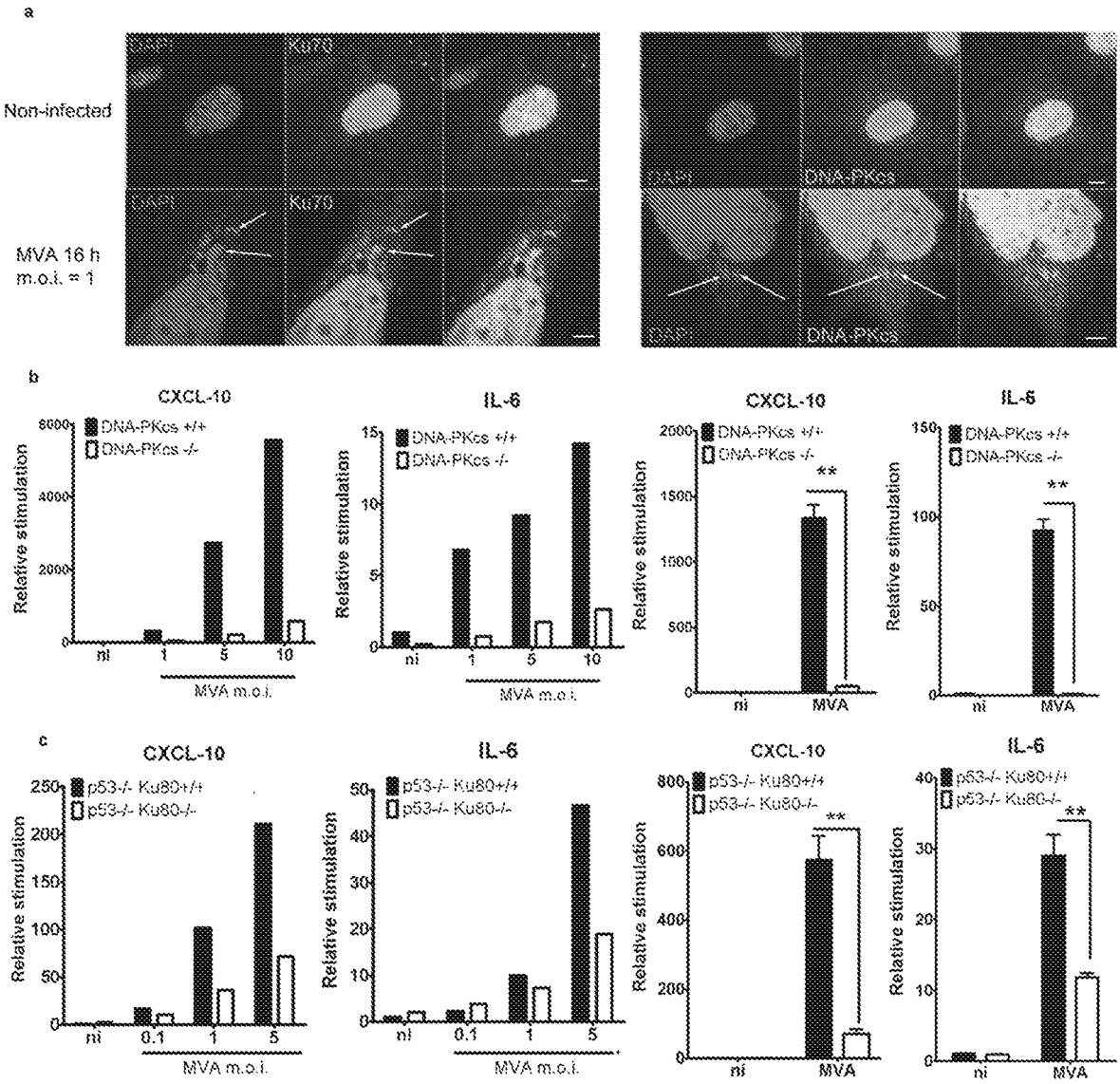

a
Non-infected
MVA 16 h
m.o.i. = 1
DAPI
Ku70
DNA-PKcs
b
CXCL-10
IL-6
DNA-PKcs +/+
DNA-PKcs -/-
Relative stimulation
MVA m.o.i.
ni
MVA
**
c
p53-/- Ku80+/+
p53-/- Ku80-/-

Figure 16

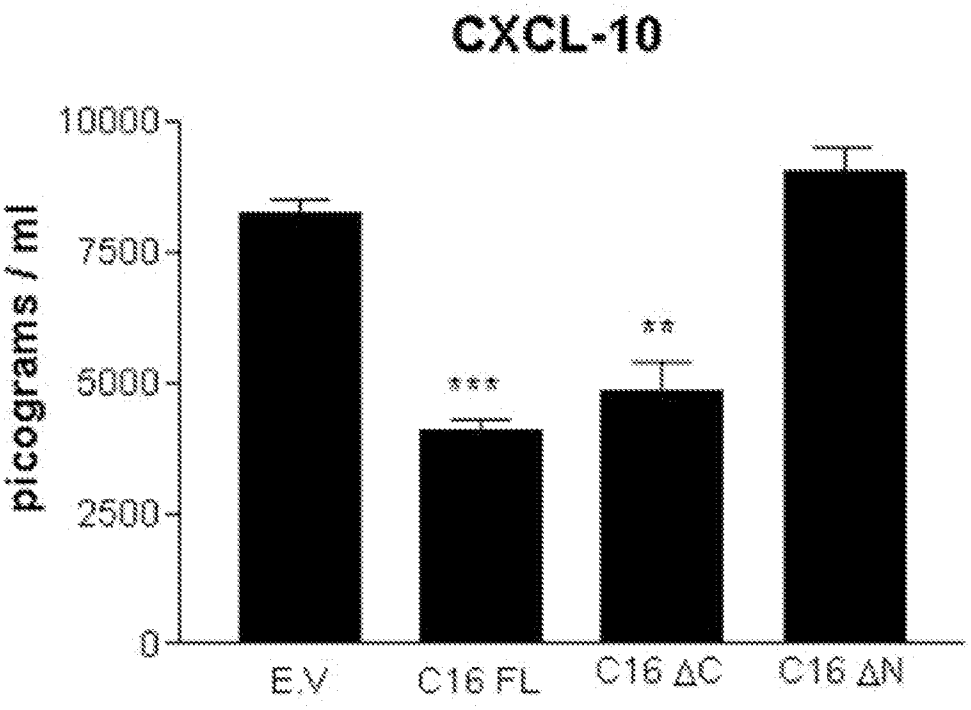

CXCL-10
picograms / ml
10000
7500
5000
2500
0
***
**
E.V
C16 FL
C16 ΔC
C16 ΔN

Figure 17

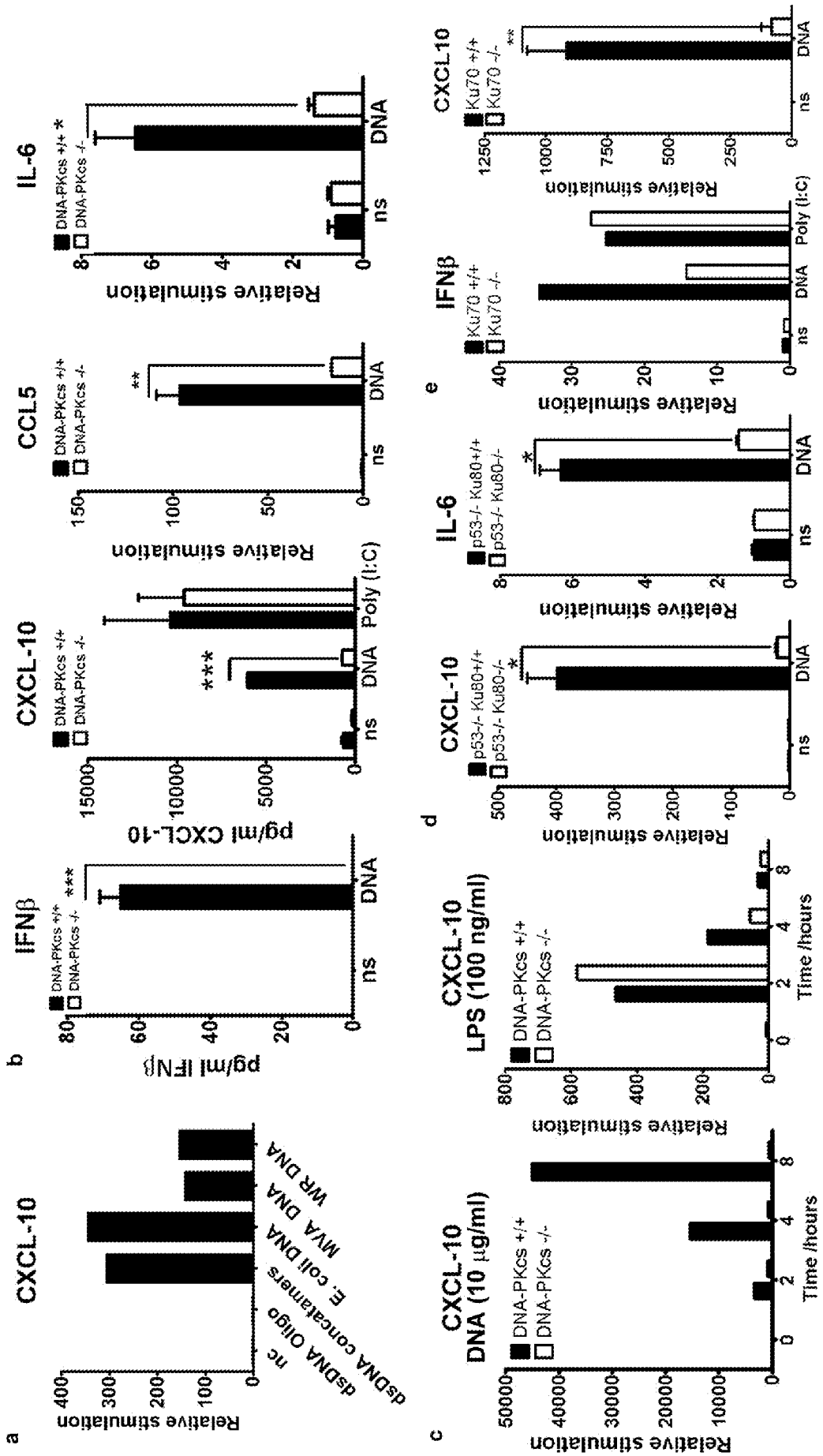

a
CXCL-10
Relative stimulation
nc
dsDNA Oligo
dsDNA concatamers
E. coli DNA
MVA DNA
WR DNA
b
IFNβ
pg/ml IFNβ
DNA-PKcs +/+
DNA-PKcs -/-
ns
DNA
CXCL-10
pg/ml CXCL-10
Poly (I:C)
CCL5
IL-6
c
CXCL-10
DNA (10 μg/ml)
LPS (100 ng/ml)
Time /hours
d
p53-/- Ku80+/+
p53-/- Ku80-/-
e
Ku70 +/+
Ku70 -/-

Figure 18a

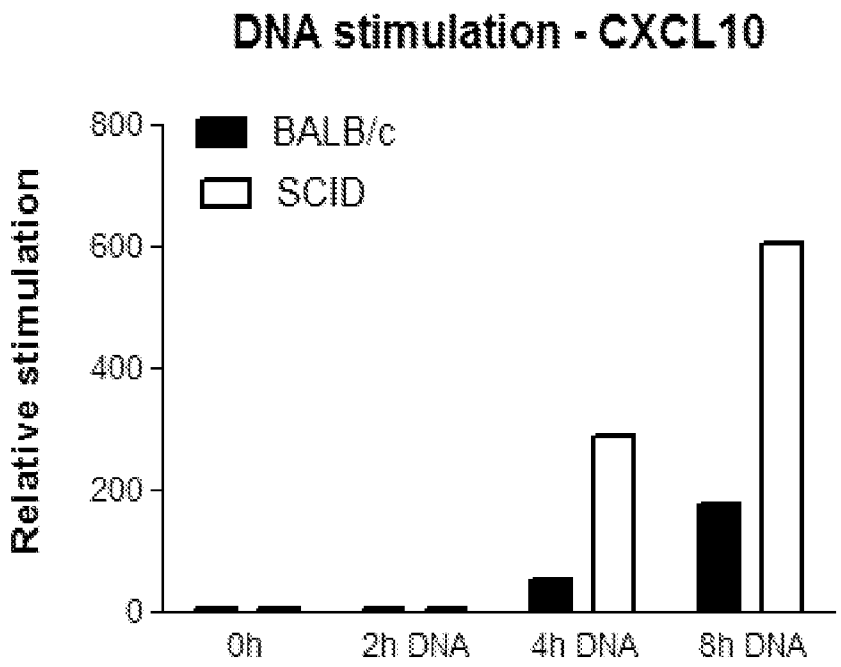


Figure 18b

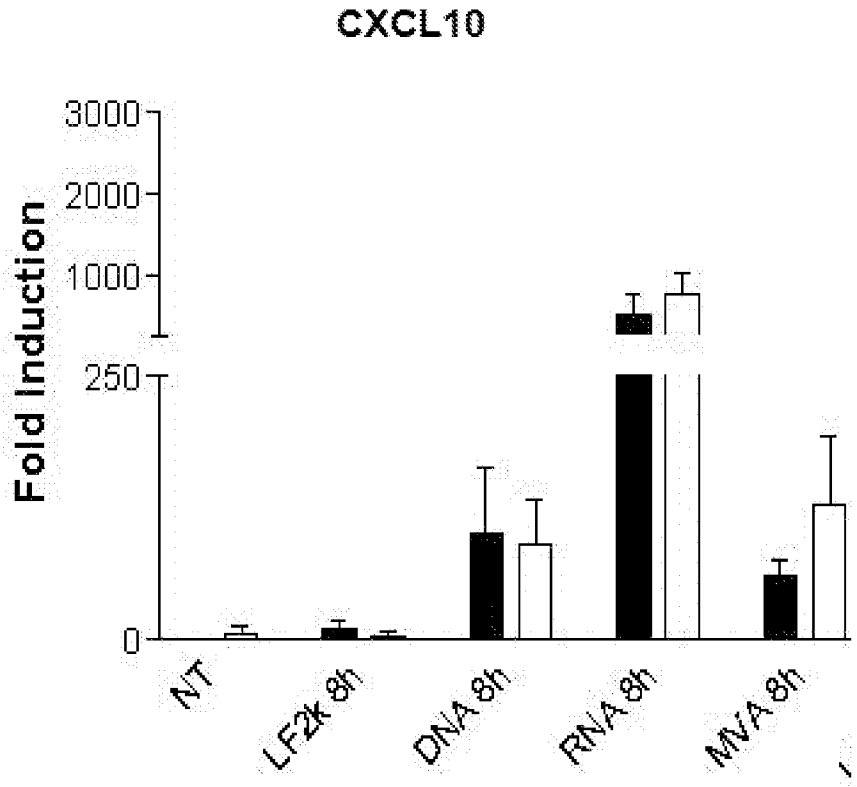
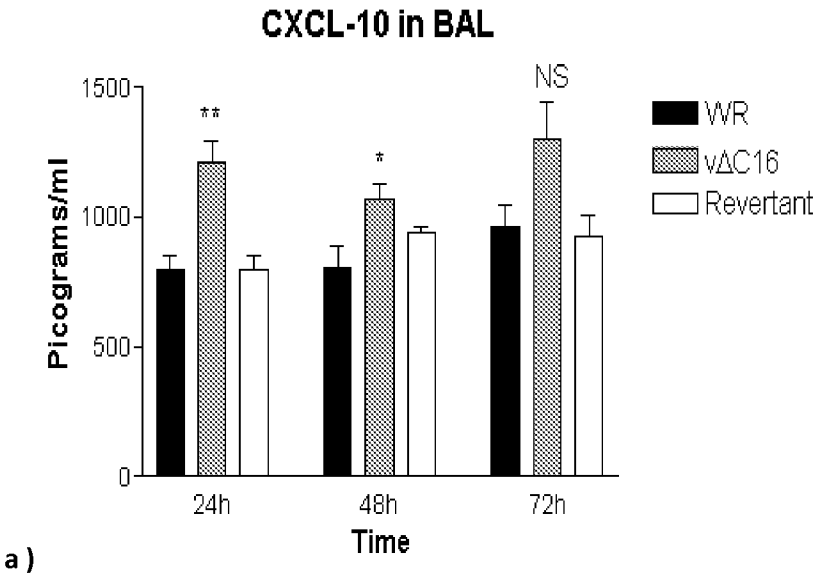
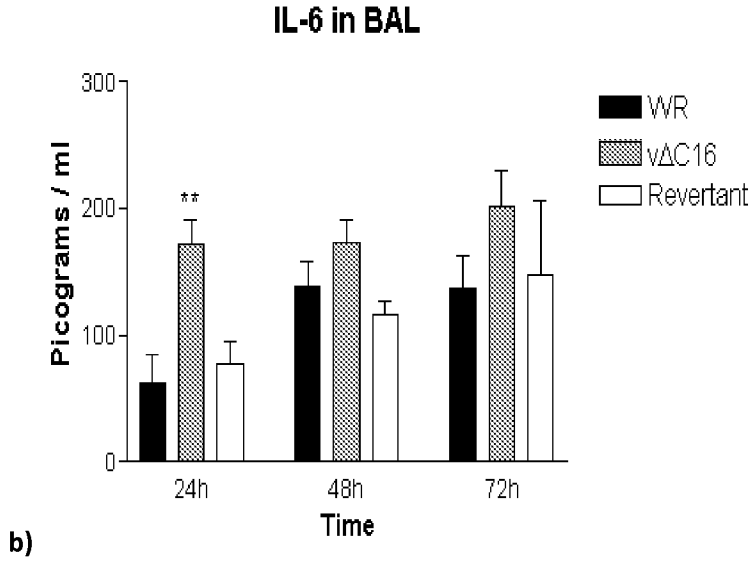


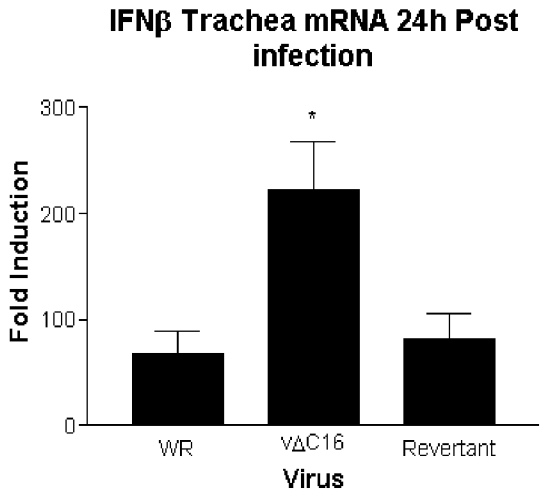
Figure 19



a)



b)



c)