The difference in IL-1β, MIP-1α, IL-8 and IL-18 production between the infection of PMA activated U937 cells with recombinant vaccinia viruses inserted 2004 H5N1 influenza HA genes and NS genes

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Summary

***Background*: The severity of avian influenza H5N1 disease is correlated with the ability of the virus to induce an over production of pro-inflammatory cytokines from innate immune cells. However, the role of each virus gene is unknown. To elaborate the function of each virus gene, the recombinant vaccinia virus inserted HA and NS gene from the 2004 H5N1 virus were used in the study.**

***Methods*: U937 cells and PMA activated U937 cells were infected with recombinant vaccinia virus inserted with HA or NS gene. The expressions of HA and NS proteins in cells were detected on immunofluorescence stained slides using a confocal microscope. The cytokine productions in the cell supernatant were quantitated by ELISA.**

***Results*: The recombinant vaccinia virus inserted with HA genes induces the production of IL-1β, MIP-1α, IL-8 and IL-18 cytokines from PMA activated U937 cells significantly more than cells infected with wild type vaccinia, whereas the recombinant vaccinia virus inserted with NS genes it was similar to that with the wild type vaccinia virus. However, there was no synergistic nor antagonistic effect of HA genes and NS genes in relation to cytokines production.**

Conclusion*: Only the HA gene from the 2004 H5N1 virus induces IL-1β, MIP-1α, IL-8 and IL-18 cytokine productions from activated U937 cells. The same HA gene effect may or may not be the same in respiratory epithelial cells and this needs to be explored. *(Asian Pac J Allergy Immunol 2011;29:349-56)

Key words: *H5N1 influenza viruses, cytokines, U937 cells, HA, NS*

Abbreviations

H5N1 influenza=		Influenza A virus subtype of hemagglutinin type 5 and neuraminidase type 1 surface antigens
HA	=	Hemagglutinin gene
NS	=	Non-structural gene
PMA	=	Phorbol myristate acetate
r-HA	=	Recombinant hemagglutinin gene
r-NS	=	Recombinant non-structural gene

Introduction

Avian influenza in humans is severe and fatal. The severity of the disease correlates with an ability of the virus to induce an over production of pro-inflammatory cytokines.[1-5] The cytokine response occurs, whereas a specific immune response has hardly been detected. Thus, a cytokine storm is the immunopathology mediated by innate immunity in H5N1 infection. Pro-inflammatory cytokines are produced in bronchial epithelial cells infected with H5N1 viruses[6] and it is also well documented that innate mononuclear cells, monocytes and macrophages, are the major cell types that release a variety of cytokines.[1,7] Information elucidated from these studies was derived from using whole virions, either wild type or reverse genetic viruses. It can not be ruled out that the interplay between different genes or their products, either RNA-RNA interaction or RNA-protein interaction, may influence certain

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gene expression and function. The only way to study individual gene function is to create a recombinant virus containing the gene of interest.

This study aimed to investigate cytokine released in culture supernatants of PMA activated U937 cells infected with recombinant vaccinia virus inserted with HA (r-HA vaccinia) or NS (r-NS vaccinia) genes from the 2004 H5N1 virus by using the ELISA technique. Confocal microscopy of immunofluorescence staining for HA or NS protein and RT-PCR for mRNA in infected cells was performed. The results show that (compared to the wild type vaccinia) chemokines; MIP-1α and IL-8, antiviral cytokine; IL-18 and proinflammatory cytokine; IL-1β are significantly released in the supernatant of PMA activated U937 cell infected with r-HA vaccinia whereas TNFα, another pro-inflammatory cytokine, is not. This effect on cytokines production is not observed for r-NS infection. This study demonstrates the direct effect of either HA or NS without any interference by other influenza genes in cytokines production in activated monocytes. Moreover, the results suggest the absence of NS gene suppressive effect and an antagonistic effect on cytokines production induced by HA gene expression.

Methods

Cell preparation

U937, a human monocyte cell line, was cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2.5 mM glutamine, 10mM HEPES, 10,000U/ml penicillin and 10,000µg/ml streptomycin. The cells were activated by incubating them with 10 nM of phorbol myristate acetate (PMA) (Sigma, Mo. U.S.A) for 24 hours (activated U937 cell).

Viruses

The viruses used in this study were: Vaccinia virus Lister strain (wild type control), recombinant vaccinia virus inserted with HA (r-HA vaccinia) and recombinant vaccinia virus inserted with NS (r-NS vaccinia). Both the HA and NS genes of the two recombinant viruses were derived from A/Thailand/1(KAN-1)/04 (H5N1) (sequences of both genes can be accessed from GenBank with accession numbers AY555150 and AY626146 for HA and NS respectively). These viruses were propagated and titrated in TK cells. Titers were presented as plaque forming units /ml.

Virus infection in U937cell culture

Resting (no PMA) or activated (with PMA) U937 cells at a concentration of $1x10^6$ cells/ml in 2% FBS RPMI 1640 were infected in triplicate with wild type vaccinia or r-HA vaccinia or r-NS vaccinia at a multiplicity of infection (M.O.I.) of 0.05 in a 96 well- tissue culture plate. An M.O.I. of 0.1 was obtained by double infection with both types of the recombinant viruses. Infection was carried out for 24 hours at 37°C in a humidifying CO_2 incubator. Thereafter, culture supernatants were harvested and kept at -80°C for cytokine assays by ELISA. Meanwhile; cell pellets were washed once with PBS and divided into two portions; one portion was used for RNA extraction by TRIzol® Reagent (Invitrogen, Carlsbad, CA., USA.) and another portion was smeared on microscopic slides for immune-fluorescence staining.

Semiquantitation for HA or NS mRNA by RT-PCR

Total RNA extracts were reverse transcribed into cDNA by using AMV reverse transcriptase™ first-strand kit (Invitrogen, Carlsbad, CA., USA.). The cDNA was further used as template in polymerase chain reactions (PCR). The primer pairs were: HA-H5f:5'-ACTCCAATGGGGGCGATAAA-3', HA-H5r:5'-CAACGGCC TCAAACTGAGTGT-3'and SNSF:5'-GATAAGGCACTTAAAATGCCG-3'and SNSR: 5'-ACGGTGAGATTTCTCCCACG-3'. The β-actin gene was amplified as the internal control 5'-ATC TGG CAC CAC ACT TCT ACA-3' as the forward primer and 5'- GTT TCG TGG ATG CCA CAG GAC T-3'as the reverse primer. Amplicons were analysed by densitometer (Amersham Biosciences, England) using Image Scanner Software (Image MasterTotal Lab version 2.01) analysis. The percentage of gene expression originating from HA and NS mRNA were semiquantitated by comparing them to that of the β-actin gene.

Immunofluorescence assay for HA or NS protein

Slides of cell deposits were air-dried and fixed in pre-cooled acetone at -20°C for 10 minutes. The slides were stained immediately, or otherwise kept at -70°C until staining by the indirect immunofluorescence technique. Goat antiserum against H5N1 HA (kindly provided by Dr. Richard Webby, St Jude Children Research Hospital) or goat anti-NS peptides antiserum (Santa Cruz Biotechnology Inc., CA., USA) was used as the primary antibody, FITC conjugated rabbit anti-goat Ig was used as the second antibody. Uninfected cells and cells infected

with wild type vaccinia virus were used as negative controls. The stained slides were observed for fluorescence using a Confocal Laser Scanning Biological Microscope (FV1000 Fluoview, Olympus, Tokyo, Japan).

Cytokine quantitation by ELISA

Culture supernatants were assayed for pro-infammatory cytokines: TNFα and IL-1β, chemokines: MIP-1α and IL-8, and antiviral cytokines: IFNα and IL-18 by ELISA kits (R&D Systems Inc., Minneapolis, USA.). The test protocols followed those described in the kit instructions. The reproducibility of the results was confirmed by repeating the experiments in culture supernatants collected from the three separate experiments.

Statistical analysis

The cytokine concentrations from ELISA (in triplicate) were analysed using Prism software (GraphPad prism 4 Software). Nonparametric, one tailed Mann Whitney u test was used; a p value < 0.05 was considered statistically significant.

Results

HA or NS proteins are more strongly expressed in PMA activated U937 cells than in resting/non-activated U937 cells

Compared to the results of semi-quantitation of mRNA for HA and NS band density of RT-PCR (% band density for HA from PMA activated U937 is 152.5 and non-activated is 96), the amount of positive immunofluorescence staining in PMA activated U937 cells was higher and more intense than in non-activated U937 cells as shown in Figure 1A and 1B for anti HA staining, whereas there was negative staining in wild type vaccinia infected cells.

A similar pattern is observed for anti NS staining as shown in Figure 2A and 2B (% band density for NS from PMA activated U937 is 41.7 and non-activated is 12.4).

Cytokine productions in resting/non-activated U937 cell

There were no differences in cytokines production from non activated U937 cells infected with r-HA vaccinia or r-NS vaccinia or both or wild vaccinia. Three groups of cytokine effector functions were investigated; these are pro-inflammatory cytokines (TNFα and IL-1β), chemokines (MIP-1α and IL-8) and antiviral cytokines (IFNα and IL-18). The concentrations of these 6 cytokines in the

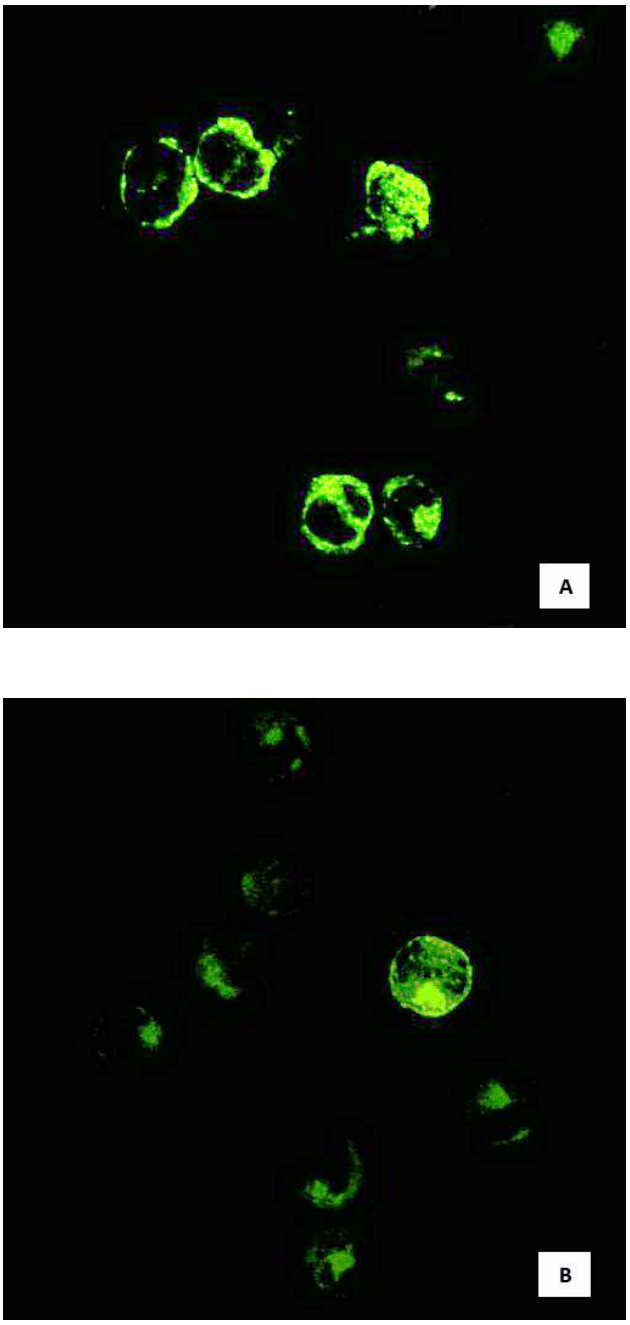


Figure 1. A. Anti HA immunofluorescence staining of PMA activated U937 cells infected with recombinant HA vaccinia (r-HA vaccinia), observed by UV light under a confocal microscope (400 magnification).
B. Anti HA immunofluorescence staining of U937 cells infected with recombinant HA vaccinia (r-HA vaccinia), observed by UV light under a confocal microscope (400 magnification).

supernatants of non activated U937 cells were not different in all infections, the average cytokine concentrations from 3 separate infections (n=3) of r-HA, r-NS and dual vaccinia infections were not different from wild type vaccinia infection.

Cytokine production in activated U937 cell

Cytokine production from r-HA vaccinia or r-NS vaccinia or both infected PMA activated U937 cells were different. The cytokine concentrations in the supernatants of infected PMA activated U937 cells were different in rHA vaccinia, rNS vaccinia or dual infections. rHA vaccinia infection induces increased production of TNF α, IL-1 β (significantly), MIP-1 α (significantly), IL-8 (significantly) and IL-18, compared to wild vaccinia infection. On the contrary, the rNS vaccinia infection did not induce any effect. The cytokine concentrations in rNS vaccinia infection supernatants were the same as in wild vaccinia infection. Furthermore, in supernatants of the dual infections with rHA and rNS vaccinia, the TNF α, IL-1 β, MIP-1 α and IL-18 levels were significantly higher than in the supernatant of wild vaccinia infection, but the levels of the cytokine concentrations were almost the same as observed in supernatants of rHA vaccinia infections, as shown in Figure 3A-F.

Discussion

Cytokine dysregulation is proposed to be a mechanism explaining the unusual severity of avian influenza. High levels of inflammatory cytokines such as TNFα , IL-1 and IL-6 or chemokines such as IL-8 and CCL2 were detected in the patients' serum.[2,3,5] *In vitro* studies had demonstrated hyper-production of pro-inflammatory cytokines in macrophages infected with H5N1 viruses.[1] Excessive infiltration of macrophages and neutrophils together with significantly higher levels of pro-inflammatory cytokines were noted in the lungs of mice infected with 1918 H1N1 virus and in those had had recent H5N1 virus infection with A/Thailand/SP/83/2004 and A/Thailand/16/2004.[8] Based on the knowledge that macrophages could be infected by H5N1 viruses and macrophage is the major source of innate cytokine, the present study used U937 as the cell source to explore the effect of the H5N1 HA gene and the NS gene, and their interaction, on cytokine production. An advantage of this study was the use of recombinant vaccinia viruses, so that the influence of the HA or NS gene could be studied

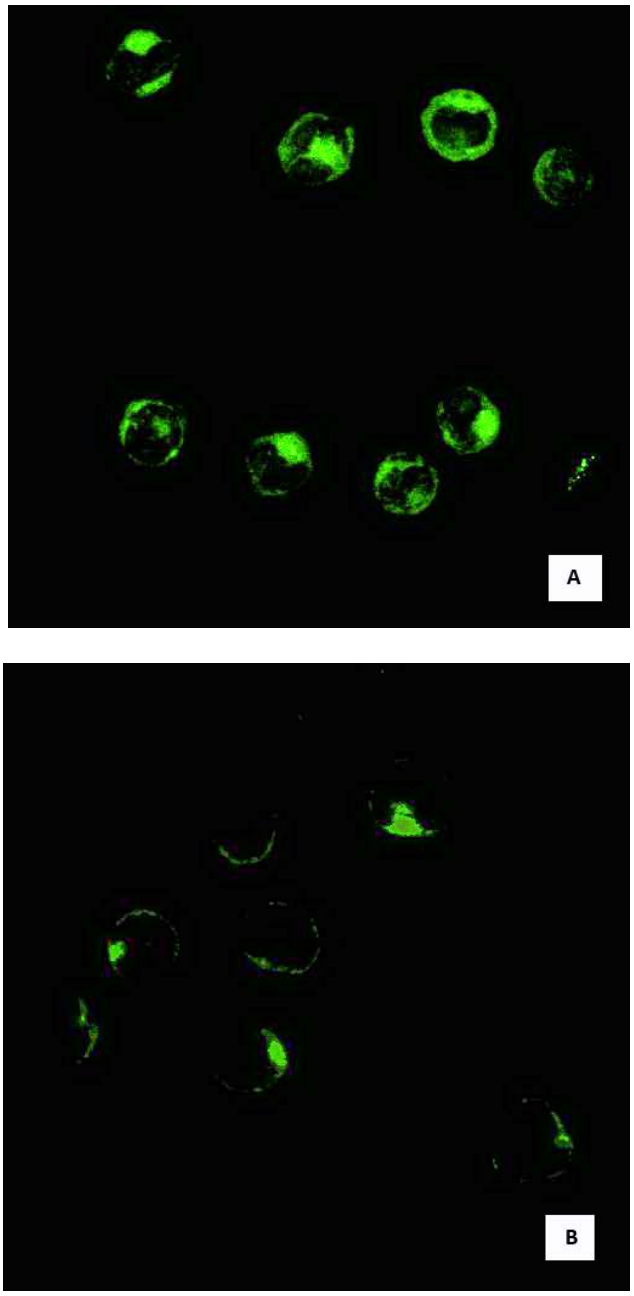
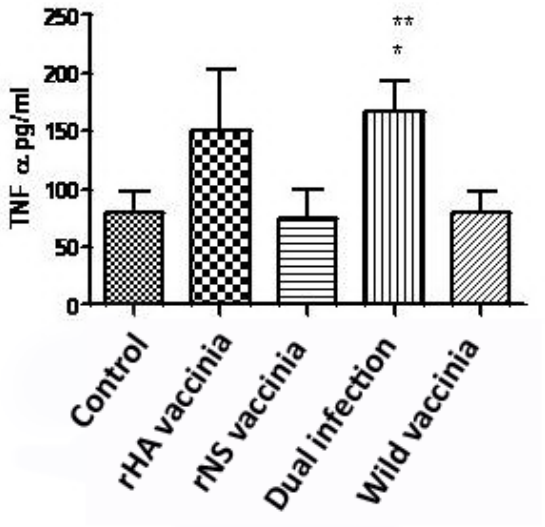


Figure 2. A. Anti NS immunofluorescence staining of PMA activated U937 cells infected with recombinant NS vaccinia (r-NS vaccinia), observed by UV light under a confocal microscope (400 magnification).
B. Anti NS immunofluorescence staining of U937 cells infected with recombinant NS vaccinia (r-NS vaccinia), observed by UV light under a confocal microscope (400 magnification).

without interference from the other influenza genes. Wild type vaccinia virus, as the parent of recombinant virus, provided the background levels of the cytokines released. In addition, using U937 cells provided advantages as well, since the essentials of the cell condition in HA or NS gene expression and cytokine production can be elucidated. The PMA stimulated U937 cells were demonstrated to exhibit increased expression of a variety of cellular molecules, including the ability to mediate chemotaxis and phagocytosis, i.e., the stimulated cells became activated and mature.[9] The present study showed that viral infection in activated U937 cells induced higher levels of influenza specific mRNA, protein expression and of all the cytokines studied, when compared with the infection in resting cells. No cell death was observed either in resting or activated cells under the conditions of infection at an M.O.I. of 0.05 or 0.1 in case of double infection for 24 hours. This study suggests that activation of U937 cells or the monocyte maturation may provide biochemical conditions that allow the HA or NS gene to exert their influence on the cytokines synthesis. This effect may or may not be the same as that of dendritic cells, which was reported by other investigators.[10] A low amount of the chemokine, MIP-1α, was detected in the cell supernatant of rHA-vaccinia infected non-activated U937 cells, which was the same level in cell supernatant of wild vaccinia infection. This is normally detected in virus infection such respiratory virus infection.[11] However, significantly increased production of both detected chemokines, MIP-1 α and IL-18 levels was observed in the cell supernatant of rHA vaccinia infected activated U937 cells (Figure 3C and 3D). This may suggest that chemokines are cytokines produced to influence the immune cell recruitment to influenza infected cells. TNFα is a cytokine that plays this role or functions as "intracrine".[12] TNFα probably plays this role in H5N1 influenza infection too. Others have reported the hyper-production of TNFα in human macrophages infected with the 1997 H5N1 virus and the high production of MIP-1α, IL-1α, KC (mouse equivalent to human IL-8), IL6, MCP-1 and IFN-γ lung of mice infected with 1918 influenza virus or recent H5N1 virus.[8] Our results show non-significant production of TNFα in 24 hours the supernatant of rHA-vaccinia infection in activated U937 cells but significant production of MIP-1α and IL-8, Figure 3A, 3C, 3D. This may indicate that during the 24 hr period of rHA-vaccinia infection TNFα effected chemokines induction and was then used as an autocrine. The cytokine storm is hypothesized to be the major cause of the unusual disease severity in avian influenza and during that period of time TNFα should be produced from dendritic cells; however, cytokine inhibition did not protect mice against lethal challenge with H5N1 virus.[13]

In contrast to the increase in cytokines production in rHA-vaccinia infection, this study demonstrated that rNS-vaccinia was a poor cytokine inducer. Levels of cytokines induced by rNS-vaccinia were not significantly different from those induced by wild type vaccinia virus. Moreover, the levels of cytokines synthesized in cells infected with both rHA-vaccinia and rNS-vaccinia, the dual infection, were not different from those released from cells infected with rHA-vaccinia. This result implies that the NS gene transcription and translation processes do not exert any antagonistic nor synergistic effect in cytokine induction mediated by processes in the HA gene, at least in activated U937 cells. The first H5N1 avian influenza outbreak in 1997 caused the disease in 18 human cases with six deaths, or a fatality rate of 33%.[14] The current H5N1 avian influenza epidemic is even more disastrous and covers a wide geographical area. Even though cytokine dysregulation is proposed to be the mechanism of disease severity in patients from both outbreaks, the NS protein of H5N1 viruses that caused the recent outbreak does not possess Glu92, but Asp92 instead. Nevertheless, the recent viruses possess Ala149.[14] Role of Glu92 and Asp149 on the virulence and pathogenicity of avian influenza are in doubt as a result of the study done by Perrone, et al.[8] The study involved two recent isolates from fatal cases: A/Thailand/16 and A/Thailand/SP/83, which both possess Glu92 and Asp149.[8,15] A/Thailand/16 was highly virulent in the mouse model with a mouse lethal dose 50 (MLD50) of 1.7, while A/Thailand/SP/83 was of low virulence with an MLD50 of 5.5.[8] These previous studies together with our results have led to the suggestion that the NS1 gene alone is not enough to elicit cytokine dysregulation. Moreover, the interplay between HA and NS genes in double infected cultures did not lead to an enhancement of cytokine synthesis. The results of the present study suggests that the severity of avian influenza might be derived from the interplay between genes, apart from HA and NS genes, within cassette of the viral genome. Essentially, this study demonstrated an important role for HA in certain cytokine inductions.


TNF α pg/ml
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Control
rHA vaccinia
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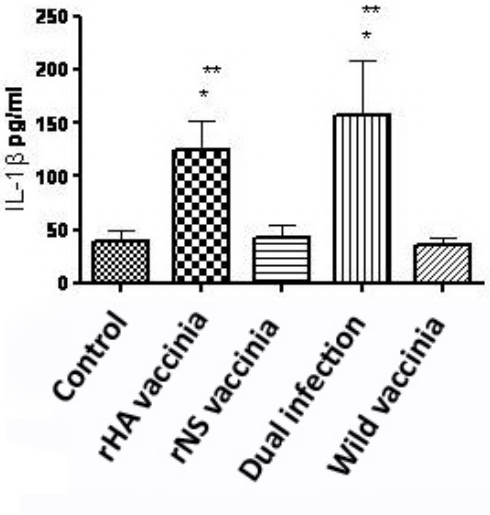
Dual infection
Wild vaccinia

A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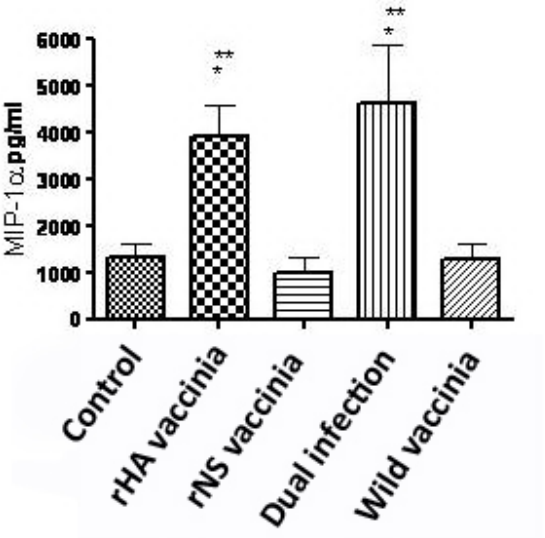
Control
rHA vaccinia
rNS vaccinia
Dual infection
Wild vaccinia

B


MIP-1α pg/ml
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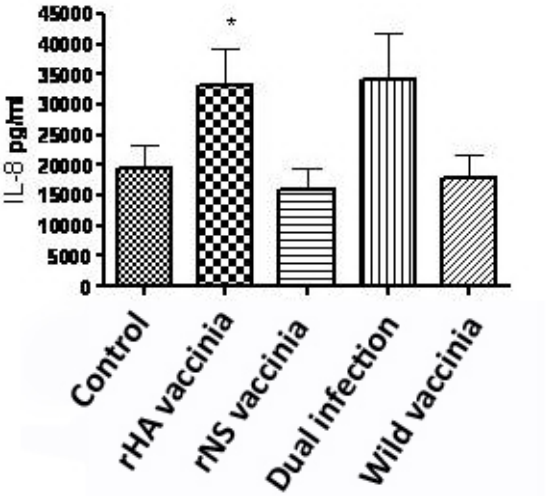
Wild vaccinia

C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Dual infection
Wild vaccinia

D

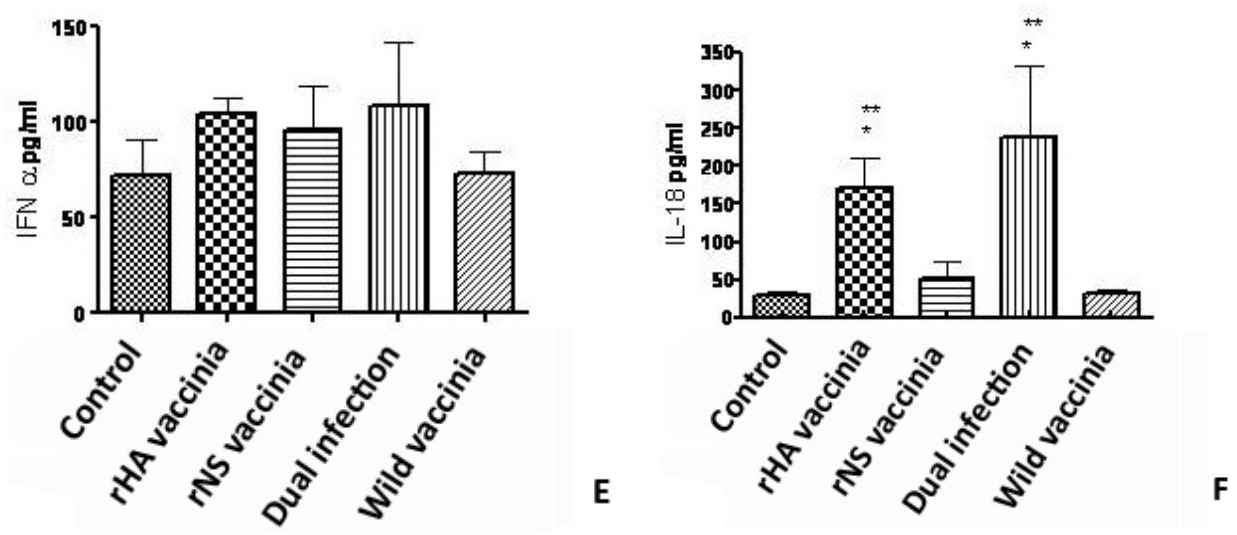

Figure 3. A. Histogram shows TNFα cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

B. Histogram shows IL-1β cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

C. Histogram shows MIP-1α cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

D. Histogram shows IL-8 cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

E. Histogram shows IFNα cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

F. Histogram shows IL-18 cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

*statistically significant against wild vaccinia infection,

**statistically significant against non-infected control (PMA activated U937 cell).

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