Cell-free chromatin from dying cells trigger an immune response *via* the cGAS-STING pathway

Authors:

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Abstract: Understanding what triggers an immune response is the central unresolved issue in immunology, although the association between immune response and cell death has long been recognized. Here, we show that cell-free chromatin particles that are released from dying cells, or those that circulate in blood, can readily enter healthy human lymphocytes to activate CD69 expression and the release of inflammatory cytokines. The latter can be abrogated by the STING protein inhibitor H151, indicating that cell-free chromatin particles activate an immune response via the cGAS-STING pathway. Cell-free chromatin particles released from dying cells may represent the missing link between cell death and immune response.

One-Sentence Summary: Cell-free chromatin particles released from dying cells could be the missing link between cell death and immune response.

Main Text:

Understanding what triggers an immune response is the central but unresolved issue in immunology, although there is a well-established association between immune response and cell death **(*1*)**. Cell death results in the release of cellular components that act as damage-associated molecular patterns (DAMPs) to activate an immune response **(*2–5*)**. Optimal levels of DAMPs in healthy individuals are required to maintain immune homeostasis; however, in pathological conditions such as sepsis, cancer, neurodegenerative disorders, and auto-inflammatory as well as autoimmune diseases, excessive cell death leads to an uncontrolled release of DAMPs that can provoke hyper-inflammatory responses **(*4–6*)**. Despite intensive research efforts, the precise nature of DAMPs continues to remain obscure, hindering the development of effective therapies for life-threatening disorders **(*7–9*)**.

We previously reported that cell-free chromatin particles (cfChPs) that are released from dying cells, or those circulating in blood, are readily internalized by healthy cells where they induce DNA damage and inflammation **(*10*, *11*)**. We have also shown that cfChPs are the key triggers for multiple disorders associated with acute and chronic inflammation, such as toxicity from chemo- and radiotherapy, sepsis, ageing, and cancer **(*12–18*)**. Since inflammation is a critical component of an immune response, these observations led us to hypothesize that cfChPs released from dying cells are the elusive agents that could link cell death with an inflammatory immune response **(*19*)**. In pursuit of this hypothesis, we utilized two experimental models: 1) a co-culture system wherein peripheral blood mononuclear cells (PBMCs) were exposed to conditioned medium (CM) containing cfChPs released from hypoxia-induced dying PBMCs, and 2) direct treatment of PBMCs with serum derived cfChPs. The immunogenic effects of cfChPs were investigated by

evaluating three integral aspects of an inflammatory immune response viz. 1) immune cell activation characterized by CD69 expression on T cells and NK cells, 2) cellular stress responses, and 3) activation of inflammatory cytokines.

Results

cfChPs released from dying cells are readily internalized by PBMCs

As a first step, we revisited our prior finding that cfChPs released from dying cells are readily internalized by mouse fibroblast cells ***(11)***. We confirmed that cfChPs released from dying PBMCs are also internalized by healthy PBMCs. To demonstrate this, PBMCs were dually fluorescently labelled in their DNA and histones using bromodeoxyuridine (BrdU) and histone-2B-GFP, respectively (see Materials and Methods), and induced to undergo cell death by hypoxia. The CM of the dying PBMCs, when added to fresh PBMCs, showed copious accumulation of fluorescently dually labelled cfChPs in their nuclei at 4 h **(fig. S1).**

Immunogenic effects of CM containing cfChPs

We next investigated the possible immunogenic effects of cfChPs released from dying PBMCs by evaluating CD69 expression, cellular stress-related markers, and cytokine release by treating fresh PBMCs with hypoxic CM. To determine the optimum volume of CM required, a dose-response analysis was initially performed, which showed that maximum CD69 expression on T- and NK-cells was achieved with 200 µL CM **(Fig. 1A).** All further experiments were therefore performed using 200 µl CM. Since stress-related proteins interact with, and regulate, signaling intermediates involved in an immune response, we next investigated whether cfChPs from dying cells would activate the cellular stress-related markers c-Jun, c-Fos, Jun-B, FosB, EGR-1, and NF-ĸB. Activation of stress markers was evaluated utilizing qRT-PCR in a time course experiment, which

showed upregulation of all six markers albeit at different time points **(Fig. 1B)**. The treatment of PBMCs with 200 µL CM was also associated with marked activation of the inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, and CXCL9 **(Fig. 1C).**

Serum derived cfChPs are readily internalized by PBMCs

Since we previously reported that cfChPs isolated from human sera are readily internalized by mouse fibroblast cells **(*10*)**, we speculated, whether serum derived cfChPs, like those contained in hypoxic CM, might also activate inflammatory cytokines? As a first step, we investigated whether serum derived cfChPs are internalized by PBMCs. We labelled the DNA and histones of serum derived cfChPs with Pt Bright 550 (red) and ATTO-488 (green; see Materials and Methods), respectively. When the dually labelled cfChPs (10 ng) were added to the fresh PBMCs, co-localizing red and green fluorescence signals representing cfChPs were clearly detected in their nuclei with optimal accumulation observed at 2 h (**fig. S2**).

Immunogenic effects of serum derived cfChPs

To investigate whether serum derived cfChPs, like those released from dying cells, would trigger an immune response, we treated PBMCs with serum derived cfChPs (10 ng) and evaluated their immunogenic potential. The treatment of PBMCs with cfChPs triggered a marked increase in the expression of CD69 on T-cells and NK-cells at 24 h **(Fig. 2A).** cfChPs also activated NF-KB, c-Jun, c-Fos, Jun-B, FosB, and EGR-1 on human PBMCs, but at different time points **(Fig. 2B)**. A pronounced increase in levels of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, CXCL9, and IFN-β **(Fig. 2C)**, as well as the two anti-inflammatory cytokines IL-10 and TGF-β1 **(Fig. 2D)**, was also detected.

Serum derived cfChPs act via the cGAS-STING pathway

Since the cGAS-STING pathway is activated when DNA is atypically present in the cytoplasm **(*20*),** we hypothesized that the internalized cfChPs may activate cGAS-STING

while in the cytoplasm. The treatment of PBMCs with serum derived cfChPs (10 ng) led to upregulation of STING and cGAS mRNA expression which was maximally seen at 12 h, thereby corroborating our hypothesis **(Fig. 3A)**. Importantly, prior treatment of PBMCs with the STING protein inhibitor H151 virtually abolished cfChP-induced cellular stress mRNA expression **(Fig. 3B)**. Prior treatment with H151 also highly significantly reduced production of inflammatory cytokines as estimated by flow cytometry **(Fig. 3C)**. These findings strongly suggest that activation of the cGAS-STING pathway is the underlying mechanism by which cfChPs activate an immune response.

Discussion

We made several notable observations in this study. First, cfChPs released from dying PBMCs, as well as serum derived cfChPs, rapidly enter immune cells and accumulate within their nuclei. Second, cfChPs from both sources activate CD69 expression on T cells and NK cells. Third, cfChPs from both sources activate cellular stress-related pathways and inflammatory cytokines. Fourth, cfChP-induced cellular stress and inflammatory cytokine release are mediated via the cGAS-STING pathway.

Our claim that cfChPs activate immune response via the cGAS-STING pathway can be criticized on the ground that we have solely dependent on H151 induced inhibition of inflammatory cytokines to make our claim. Further studies involving pathway analysis could have elucidated the mechanism(s) involved in activation of cGAS-STING. While such mechanistic studies need to be conducted in the future, our results nonetheless represent the first report which implicates cfChPs released from dying cells as the possible missing link between cell death and immune response.

It is currently believed that cell death results in the release of DAMPs, which are responsible for the activation of an inflammatory immune response **(*4–6*)**; however, the precise nature of DAMPs continues to remain elusive **(*7–9*)**. The structural diversity of DAMPs is striking as they comprise a wide range of molecules derived from the extracellular matrix as well as nuclear components of dying cells **(*9*)**. DNA and histones are well-known DAMPs **(*21*, *22*)**. Individually, both isolated DNA and histones exhibit various immune-stimulatory effects *in vitro* and *in vivo*. DNA induces signaling via toll-like receptor 9 (TLR9) and intracellular nucleic acid sensing mechanisms, while histones use TLR2 and TLR4 to induce cytotoxicity and pro-inflammatory signaling **(*23*, *24*)**. However, it has been argued that cell death results in the release of mono- and oligo-nucleosomes (cfChPs) rather than DNA or histones **(*25*, *26*)**. The presence of nucleosomes in the circulation can be readily detected by enzyme-linked immunosorbent assay **(*27*)**, but techniques used for the detection of dsDNA and naked histones do not assure that these molecules exist in their free forms **(*21*)**. Therefore, the existence of circulating cfDNA and free histones, and their independent immune-stimulatory effects, remains questionable **(*21*)**. Our results suggest that cfChPs, which are naturally released following cellular death, are the key triggers that activate an immune response thereby uncovering a link between cell death and immunity that has long been a vexing problem. That H151, a STING protein inhibitor, could abrogate inflammatory cytokine production strongly indicates that cfChPs activate an immune response via the cGAS-STING pathway.

We have shown, in several studies reported elsewhere, that the various pathological activities of cfChPs can be abrogated by agents that deactivate cfChPs **(*12–18*)**. Of these, the pro-oxidant combination of the commonly used nutraceuticals resveratrol and copper (R-Cu) holds the most promise for clinical application because of its lack of toxicity and

low cost. We have reported that R-Cu can ameliorate sepsis in a pre-clinical model **(*16*)** and prevent death from severe coronavirus disease 2019 in humans **(*28*)**. For this reason, R-Cu may be worthy of evaluation in clinical trials involving life-threatening disorders associated with hyper-inflammation, including sepsis, cancer, neurodegenerative disorders, and auto-inflammatory as well as autoimmune diseases.

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Investigation: S.S., N.R., R.N., R.L., D.K., N.K.K., G.V.R.
Visualization: S.S., I.M.
Funding acquisition: I.M.
Supervision: I.M.
Writing – original draft: S.S., I.M.
Writing – review & editing: S.S., I.M.
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Supplementary Materials

Materials and Methods

Figs. S1 to S2

Legends to figures

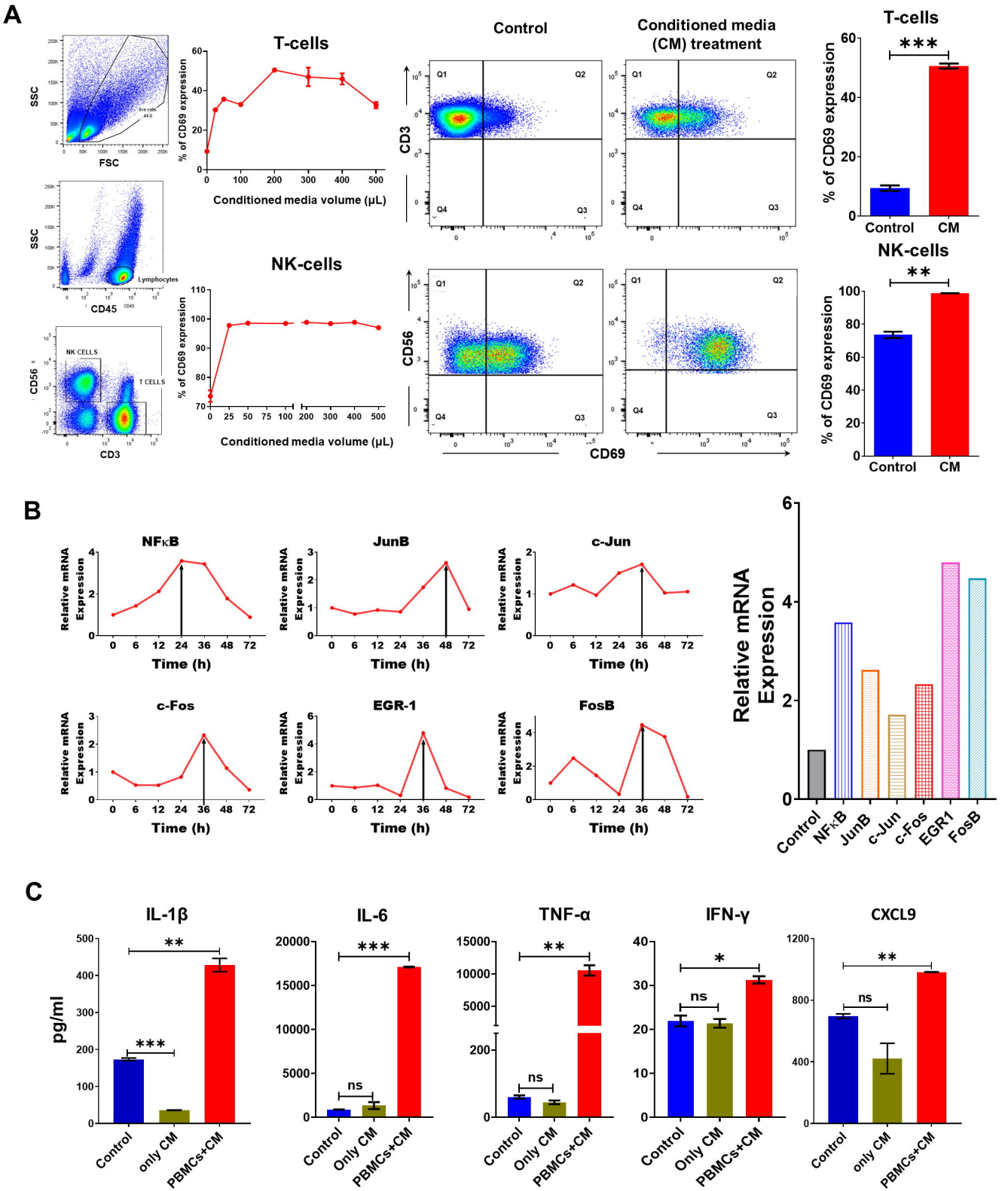
Fig. 1. cfChPs released from dying PBMCs into conditioned medium (CM) induce an immune response. (A) Representative flow cytometry plots of CM-treated PBMCs showing the gating strategy (left-hand panel). Line graphs represent the expression of CD69 in CM-treated PBMCs through dose-response analyses along with representative flow cytometry plots (middle panel). Histograms represent the relative upregulation of CD69 expression on T-cells and NK-cells using 200 µL CM (right-hand panel). **(B)** Upregulation of stress-related markers in human PBMCs following treatment with CM (200 µl). mRNA expression of six stress-related markers in PBMCs detected by qRT-PCR and analyzed using a comparative C_T method. Line graphs represent the results of time course analyses calculated as expression fold [$2^{(-\Delta\Delta C_T)}$]. Histograms represent the relative mRNA expression of stress-related markers at respective peak time points. **(C)** Histograms to represent the relative production of inflammatory cytokines by PBMCs in response to CM (200 µl) as estimated by flow cytometry. Histograms represent mean ± SEM values. Statistical analyses were performed using two-tailed unpaired student's *t*-tests in GraphPad Prism 8. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Fig. 2. Serum derived cfChPs activate an immune response. (A) Representative flow cytometry plots of cfChP (10 ng)-treated PBMCs showing gating strategy (left-hand panel). Line graphs represent the expression of CD69 in a time course experiment (blue line represents untreated cells; red line represents cfChP-treated cells) along with representative flow cytometry plots (middle panel). Histograms represent the relative upregulation of CD69 expression on T-cells and NK-cells in response to cfChPs. **(B)** Upregulation of stress-related markers in human PBMCs following treatment with cfChPs (10 ng). mRNA expression of six stress-related markers in PBMCs was detected by qRT-

PCR and analyzed using a comparative C_T method. Line graphs represent the results of time course analyses calculated as expression fold [2^(-ΔΔC_T)]. Histograms represent the relative mRNA expression of stress-related markers at respective peak time points. **(C** and **D)** Histograms representing the production of inflammatory and anti-inflammatory cytokines, respectively, by PBMCs in response to cfChPs (10 ng). Histograms represent mean ± SEM values. Statistical analyses were performed using two-tailed unpaired student's *t*-tests in GraphPad Prism 8. * $p<0.05$, ** $p<0.01$.

Fig. 3. Serum derived cfChPs activate immunogenic effects *via* the cGAS-STING pathway. (A) Upregulation of mRNA expression of cGAS and STING in human PBMCs following treatment with serum derived cfChPs (10 ng) detected by qRT-PCR and analyzed using a comparative C_T method. Line graphs represent the results of a time course experiment calculated as expression fold [2^(-ΔΔC_T)]. **(B)** Histograms represent relative mRNA expression of stress-related markers at respective peak time points and their inhibition by prior treatment with the STING protein inhibitor H151 (50 ng/ml). **(C)** Histograms represent flow cytometry data of inflammatory cytokines secreted by PBMCs in response to cfChPs (10 ng) and their inhibition by prior treatment with the STING protein inhibitor H151 (50 ng/ml). It is to be noted that treatment with H151 itself had no effects. Results are represented as mean ± SEM values and the data were analyzed using two-tailed unpaired student's *t*-tests in GraphPad Prism 8. * $p<0.05$, ** $p<0.01$.

Fig.1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% of CD69 expression
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CM
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CD45
Lymphocytes
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CD56
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NK-cells
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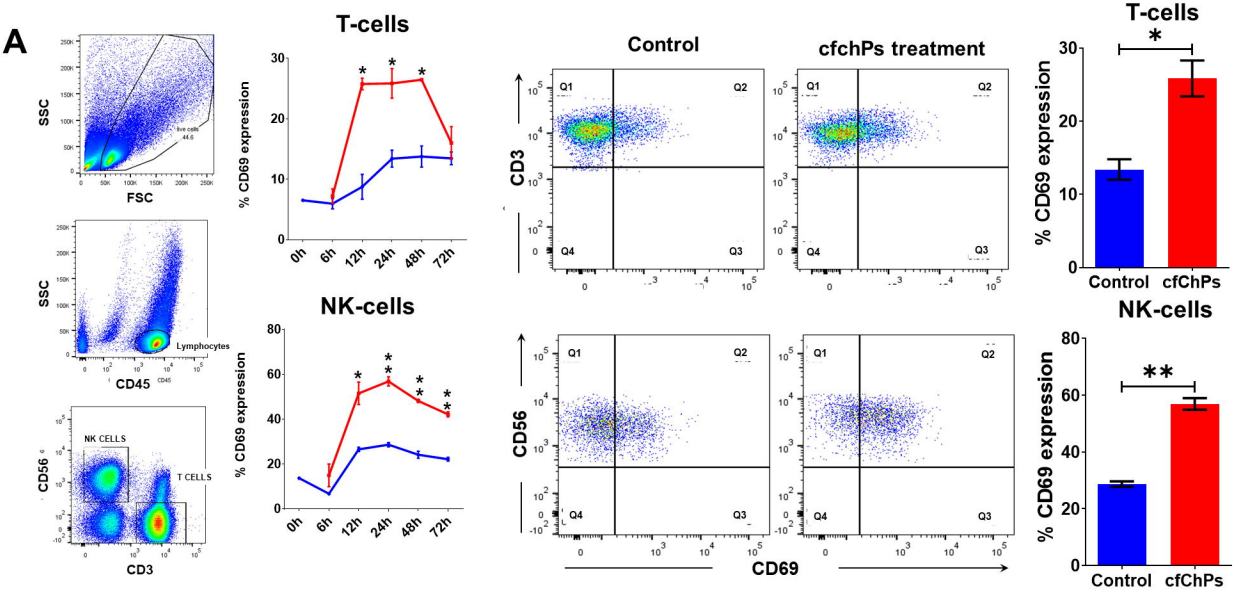
Fig.2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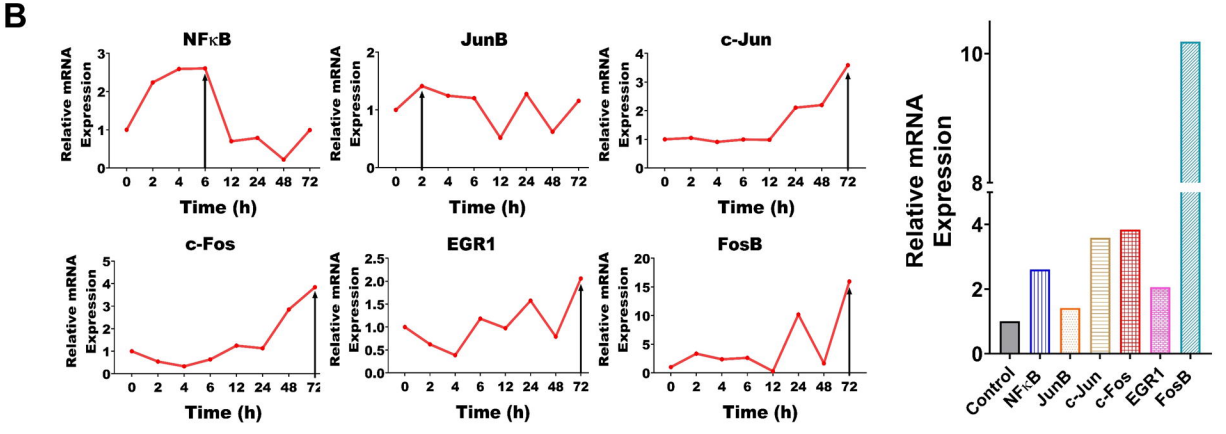
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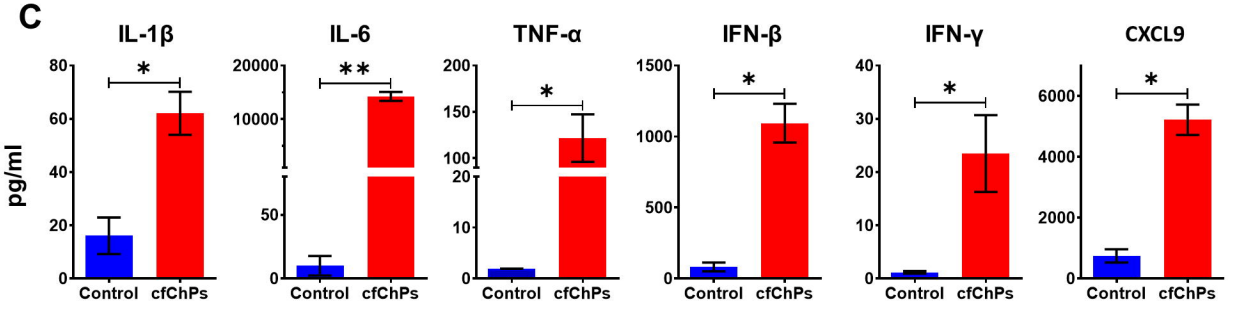
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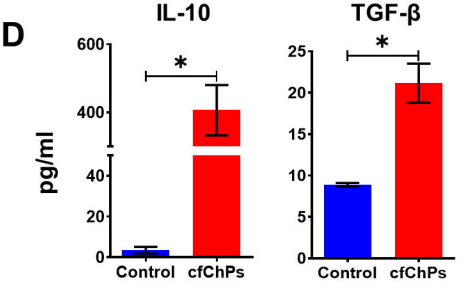
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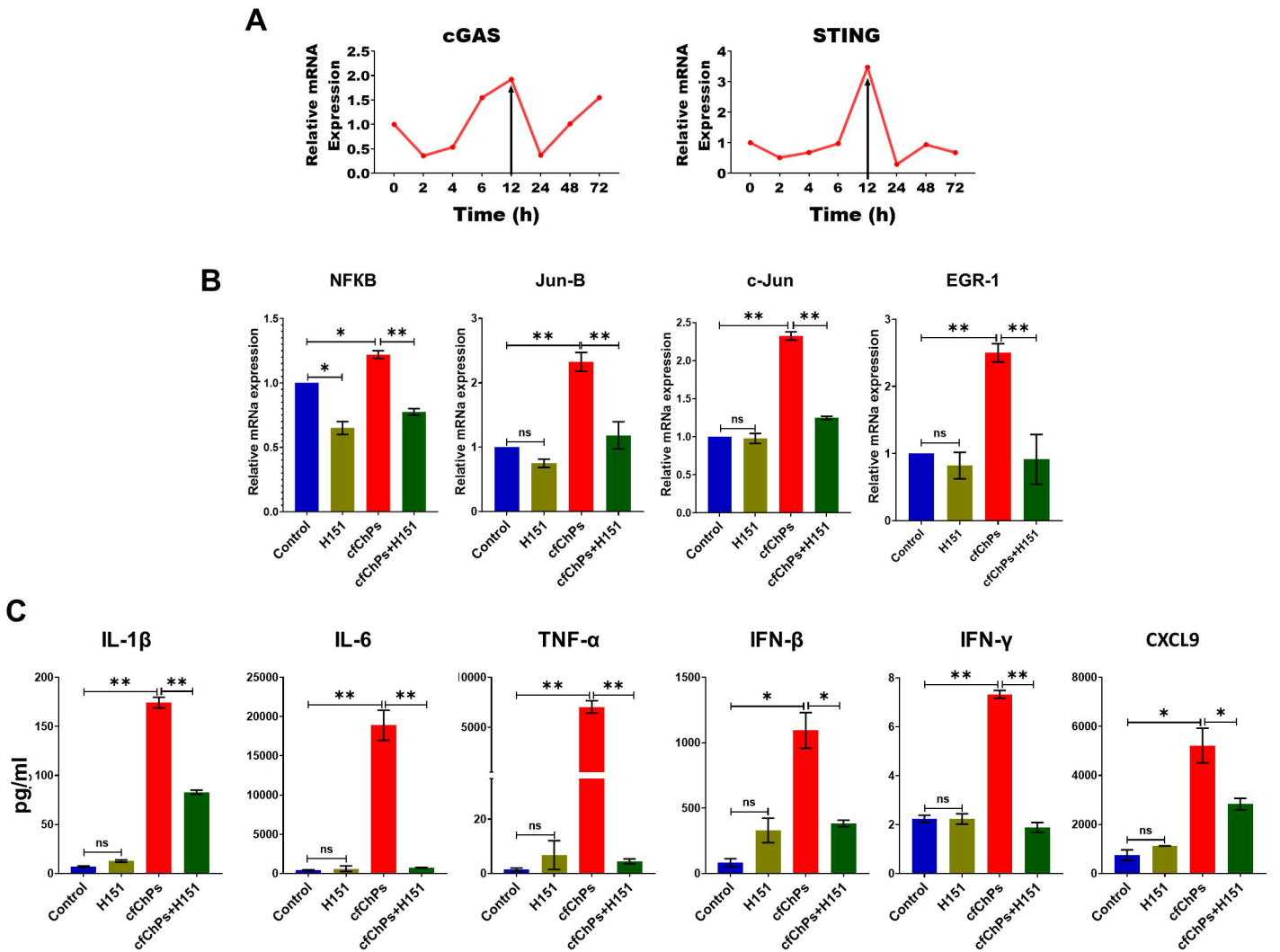
pg/ml
Control
cfChPs

Fig.3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STING
Relative mRNA Expression
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Relative mRNA expression
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H151
cfChPs
cfChPs+H151
ns
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IL-1β
IL-6
TNF-α
IFN-β
IFN-γ
CXCL9
pg/ml