Influenza Virus and Gamma Interferon Synergistically Increase Nitric Oxide Production in RAW 264.7 and AMJ2-C11 Macrophages

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Free radicals, including nitric oxide (NO), contribute to lung damage during influenza virus infection. Macrophages belong to the major cells producing NO abundantly in response to gamma interferon (IFN-γ) – a cytokine synthesized upon viral infection. However, the mechanism by which the influenza virus triggers a high-output generation of NO in macrophages is unclear. We hypothesized that influenza virus infection of macrophages synergizes with IFN-γ in NO production. For the investigation, RAW 264.7 and AMJ2-C11 murine macrophage cell lines were exposed to IFN-γ, the influenza A/PR/8/34 (H1N1) virus or a combination of both. Untreated cells served as controls. At 6 h, 12 h, 18 h and 24 h post-exposure, cell viability was evaluated, nitrite levels in macrophage culture supernatants were measured, and cells were harvested for RNA isolation and inducible NO synthase (iNOS) mRNA detection. Results revealed that the influenza virus mediated iNOS gene induction in RAW 264.7 macrophages, and the virus together with IFN-γ acted synergistically in enhancing NO synthesis in both cell lines. Furthermore, the combination attenuated cell viability more significantly than each component separately. These findings are important for explaining the conditions under which the influenza virus induces an excess production of NO in macrophages, consequently leading to a severe pulmonary inflammation.

Key words: influenza virus, gamma interferon, nitric oxide, macrophages

INTRODUCTION

Nitric oxide (NO), a gaseous free radical molecule, is produced in cells from L-arginine by one of the three isoforms of nitric oxide synthase (NOS): neuronal NO synthase (nNOS), inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) (1). Calcium (Ca^{2+}) elevation stimulates expression of nNOS and eNOS, which participate in neurotransmission and vasodilatation, respectively, whereas iNOS is independent of elevated intracellular Ca^{2+}. Proinflammatory cytokines, such as interferon-γ (IFN-γ), interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), and bacterial lipopolisaccharide (LPS) can induce iNOS at the transcriptional level in various cells, including murine macrophages (2), vascular smooth muscle cells (3), alveolar macrophages (4), mononuclear cells (5) and human lung epithelial cells (6). Noteworthy, following induction, this enzyme generates sustained and high levels of NO, especially in macrophages (7).

NO plays an immunomodulatory and antimicrobial role during infections with bacteria, viruses, protozoa, fungi. The antiviral effect of NO has been shown with the influenza virus (8), herpes simplex virus type 1 (HSV-1) as well as with other viruses (9). However, excessive production of NO can cause host tissue damage. Its contribution to lung injury and the development of pneumonia was demonstrated in experiments with mice infected with the

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influenza A virus (10) and HSV-1 (11). The response to infection involves infiltration of pulmonary tissue by macrophages and other immune cells that secrete IFN-γ. Macrophages also can produce IFN-γ as an autocrine factor (12–14). Production of IFN-γ by the various immune cells stimulates iNOS gene expression in macrophages and a high-output generation of NO, which subsequently leads to the injury of lung tissue (10). In the context of these studies, it is important to understand the mechanism by which the influenza virus contributes to the high production of NO in macrophages, and how the immune response might lead to tissue injury and disease complications well after the virus has cleaned the host.

In the present study, we investigated the hypothesis that influenza virus infection of RAW 264.7 and AMJ2-C11 macrophage cell lines *in vitro* synergizes with IFN-γ in NO production via iNOS induction. If this hypothesis is correct, there exists another mechanism/target to modify late complications of influenza infection. Otherwise, the influenza virus does not exert a direct effect on iNOS gene expression in macrophages, and NO production is dependent on IFN-γ. Also, it would suggest that the mentioned combination is not critical for a synergistic increase of NO production in macrophages, and it could be an essential condition for other cell types, particularly for epithelial cells.

MATERIALS AND METHODS

Cell cultures

All tissue culture cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA. Media and components including antibiotics for maintenance of cell cultures were purchased from Gibco Invitrogen Corp., Grand Island, NY. Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were maintained in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES buffer solution, 100 U/ml penicillin – 100 μg/ml streptomycin, 50 μg/ml gentamicin sulfate, and 1.25 μg/ml amphotericin B (Fungizone). Murine RAW 264.7 monocytes/macrophages (ATCC TIB-71) and murine AMJ2-C11 lung alveolar macrophages (ATCC CRL-2456) were maintained in Dulbecco's modified eagle medium (DMEM) with high glucose, L-glutamine, containing 10% and 5% heat-inactivated FBS, respectively, 100 U/ml penicillin – 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B (Fungizone), and 5 mM HEPES buffer solution (only for maintenance of AMJ2-C11 cells). MDCK, RAW 264.7 and AMJ2-C11 cells were cultured according to the instructions provided by ATCC and under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. In all experiments, cell viability was evaluated by Trypan blue exclusion. Digital photomicrography of macrophage cultures was performed using a camera-mounted Olympus IMT-2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) after cell treatments.

Influenza virus

The mouse-adapted influenza A/Puerto Rico/8/34 [A/ /PR/8/34] (H1N1) virus was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs as previously described (15). After harvesting, aliquots with infected allantoic fluid were stored at –86 °C until use. The influenza A/PR/8/34 virus was a kind gift of Dr. Bradley S. Bender from the University of Florida, College of Medicine, Gainesville, FL.

Determination of influenza virus infectivity titer

For the determination of the influenza A/PR/8/34 virus infectivity titer, confluent monolayers of MDCK cells were inoculated with the virus stock of serial 10-fold dilutions. The 50% tissue culture infectious dose ($TCID_{50}$) was evaluated according to the extent of viral cytopathogenic effect (CPE) in monolayers after 96 h, and the virus infectivity titer was calculated using the Kärber method (16, 17). The infectivity titer was expressed as the $TCID_{50}$/ml. In this assay, the influenza A/PR/8/34 virus infectivity titer was determined as $10^{8.4}$ $TCID_{50}$/ml.

Experimental procedure of the treatment of RAW 264.7 and AMJ2-C11 cells

RAW 264.7 and AMJ2-C11 cells were seeded at 1×10^6 cells/well in 6-well tissue culture plates containing 5 ml of serum-free DMEM/well, and they were incubated for 18 h and 6 h, respectively. After the mentioned period of time, adherent RAW 264.7 macrophages were washed three times with serum-free DMEM and were overlain with serum-free DMEM. Since AMJ2-C11 macrophage culture is mixed, that is, cells are grown in suspension and some cells are adherent, the washing of cells before treatments was not done. Then, both cell lines were treated in triplicate fashion with mouse recombinant IFN-γ [rIFN-γ] (1 ng/ml) purchased from Sigma-Aldrich Co., St. Louis, MO, and the live influenza A/PR/8/34 virus (infectivity titer 10^6 $TCID_{50}$/ml for RAW 264.7 cells and 10^7 $TCID_{50}$/ml for AMJ2-C11 cells). The experimental design for the treatments of both cell lines was the following: 1) untreated cells served as con-

trols; 2) exposure to rIFN-γ; 3) exposure to the influenza virus; 4) exposure to a combination of influenza virus + rIFN-γ. The incubation of RAW 264.7 and AMJ2-C11 cells was continued to time points of 6 h, 12 h, 18 h and 24 h in a humidified incubator at 37 °C and 5% CO_2. For these time points nitrite levels in RAW 264.7 and AMJ2-C11 cell culture supernatants were measured, and cells were harvested for RNA isolation and iNOS mRNA detection by reverse transcriptase-polymerase chain reaction (RT-PCR).

Nitrite determination

Nitrite (NO_2^-) levels, an index of cellular NO production, were determined in RAW 264.7 and AMJ2-C11 cell culture supernatants using a modified Griess reagent (Sigma-Aldrich Co., St. Louis, MO): 100 μl of cell culture supernatants were mixed with 100 μl of modified Griess reagent in 96-well plates (all samples were in triplicate) and incubated at room temperature for 15 min. Afterwards, the optical density (O. D.) at 540 nm was measured using a $PowerWave_X$ microplate reader-spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). Nitrite concentrations were calculated using the KC4 statistical program, version 2.5 (Bio-Tek Instruments Inc., Winooski, VT), by comparing O. D. of nitrite in samples with respective O. D. of standard solutions of sodium nitrite (concentrations: 0.24 μmol/ml [the lower limit of detection] to 31.25 μmol/ml [the upper limit of detection]) prepared in DMEM. For the evaluation of NO produced per viable cell, the nitrite concentrations under each treatment were divided by the cell number, and the values were expressed as NO_2^- in pmol produced per viable cell.

RNA isolation and iNOS mRNA detection by semiquantitative RT-PCR analysis

For total RNA isolation, cells in each treatment group were combined from three wells because of the reason to extract a higher quantity of cellular RNA. The total RNA was isolated using TRIZOL Reagent according to the instructions provided by Gibco Invitrogen Corp., Grand Island, NY. iNOS mRNA was detected by one step reaction using Access RT-PCR System kit (Promega Corp., Madison, WI). Based on published nucleotide sequences (18), the murine primers for iNOS mRNA detection were: 5′-GTCAACTGCAAGAGAACGGAGAAC-3′ (forward primer), 5′-GAGCTCCTCCAGAGGGTAGG-3′ (backward primer). The expected amplification product is 454 bp. Murine β-actin mRNA expression was used as an internal control. Based on published nucleotide sequences (19), the murine β-actin primers were: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward primer), 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (backward primer). The expected amplification product is 348 bp. RT-PCR was performed with 1 μg of total RNA from each sample in 25 μl reaction volumes according to the manufacturer's instructions. RT-PCR was carried out in a thermal cycler (GeneAmp PCR System 9600, version 2.01; Perkin-Elmer Applied Biosystems Corp., Norwalk, CT) under the following conditions: first strand cDNA was synthesized at 48 °C for 45 min (1 cycle) and then denaturated at 94 °C for 2 min (1 cycle), followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 1 min (annealing), and 68 °C for 45 s (extension); final extension was performed at 68 °C for 7 min (1 cycle). The amplified PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and then gels were photographed under ultraviolet transillumination. 100 bp DNA Ladder (Gibco Invitrogen Corp., Grand Island, NY) was used for identification of sizes of iNOS and β-actin amplified products. Bands corresponding to iNOS and β-actin products were quantified by densitometry using NIH Image software (by Wayne Rasband and available at http://rsb.info.nih.gov/nih-image/), and values (relative O. D. units) are given as the iNOS/β-actin ratio.

Statistical analysis

Data are expressed as means ± standard error of mean (SEM). Statistical evaluation was performed with SPSS program, version 10.0, using independent samples *t*-test for comparison of means. A p value less than 0.05 was considered statistically significant.

RESULTS

Viability of RAW 264.7 and AMJ2-C11 macrophages during experiments

The viability of untreated RAW 264.7 and AMJ2-C11 macrophages at all time points was ≥ 96%. Treatment with rIFN-γ did not affect the viability of either cell line compared with controls (Fig. 1A and B). From 6 h to 24 h, the viability of RAW 264.7 macrophages declined from 95% to 70 ± 2% with influenza virus treated cells, and from 95 ± 1% to 56 ± 2% for combination of influenza virus + rIFN-γ (Fig. 1A). The viability of AMJ2-C11 macrophages fell from 95 ± ± 1% to 69 ± 1% with influenza virus, and from 93 ± ± 1% to 45 ± 2% with influenza virus + rIFN-γ combined treatment (Fig. 1B). In both cases, treatment with the combination of influenza virus + rIFN-γ re-

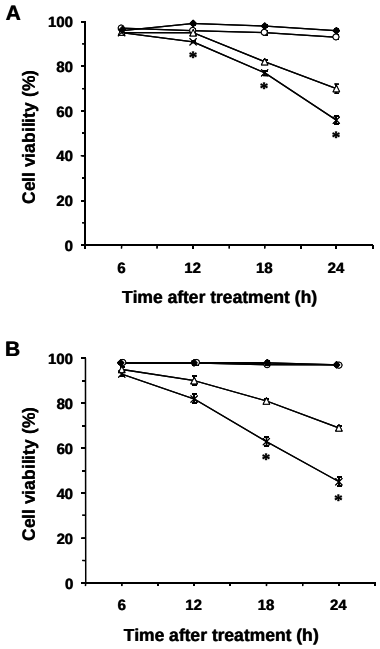

Fig. 1. Viability of RAW 264.7 (A) and AMJ2-C11 (B) macrophages at 6 h, 12 h, 18 h and 24 h after exposure to rIFN-γ (O), influenza virus (Δ) or a combination of influenza virus + rIFN-γ (×). The symbol of diamond (♦) represents controls (untreated cells). Values are means ± SEM of triplicates in a single experiment. $^{*}p < 0.05$ compared to rIFN-γ and influenza virus

duced the density of cells more than the treatment with influenza virus. Moreover, along with decreasing the number of cells, changes were observed in the morphology of macrophages, which were characteristic of the CPE, that is, variation in size and shape of cells as well as granulation (vacuolation), degeneration and lysis of cells (Fig. 2).

Effect of rIFN-γ on NO production and iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages

As shown in Fig. 3A and B, the exposure of RAW 264.7 and AMJ2-C11 macrophages to rIFN-γ sig-

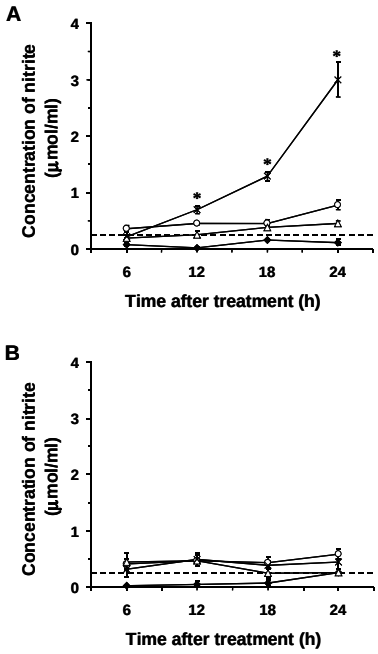

Fig. 3. Nitrite (NO_2^-) levels in supernatants of RAW 264.7 (A) and AMJ2-C11 (B) macrophage cultures at 6 h, 12 h, 18 h and 24 h after exposure to rIFN-γ (O), influenza virus (Δ) or a combination of influenza virus + rIFN-γ (×). The symbol of diamond (♦) represents controls (untreated cells). The dashed line crosses Y axis at the point of 0.24 μmol/ml indicating the lower limit of NO_2^- detection. Values are means ± SEM of triplicates in a single experiment. $^{*}p < 0.05$ compared to rIFN-γ and influenza virus

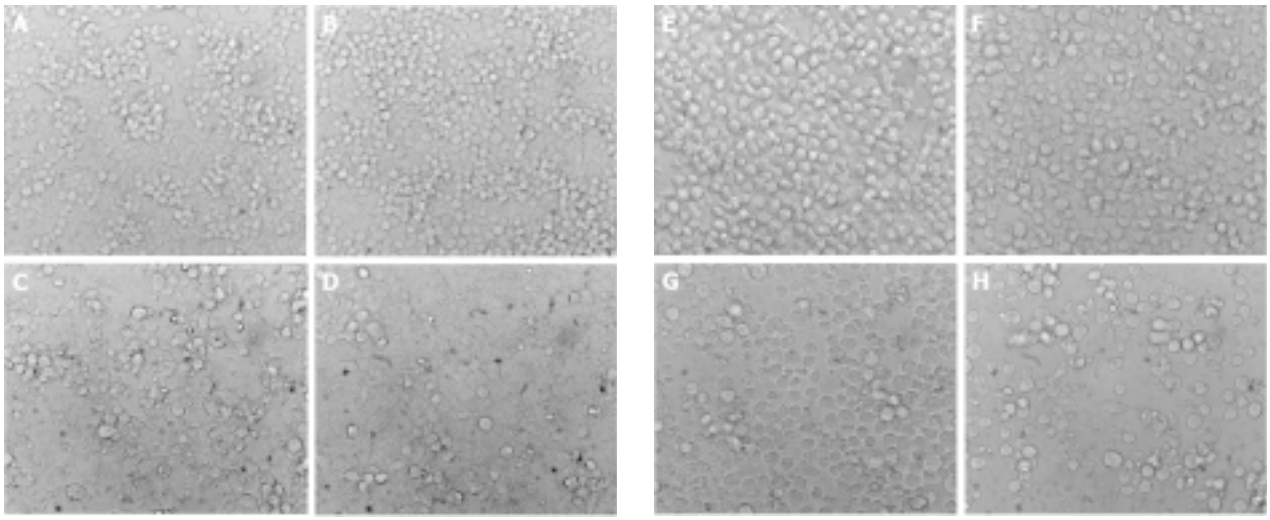

Fig. 2. Phase-contrast microscopy showing morphology of RAW 264.7 (A, B, C, D) and AMJ2-C11 (E, F, G, H) macrophages after 24 h of incubation. A and E – untreated cells (controls); B and F – cells treated with rIFN-γ; C and G – cells treated with influenza virus; D and H – cells treated with combination of influenza virus + rIFN-γ. Magnification, × 40

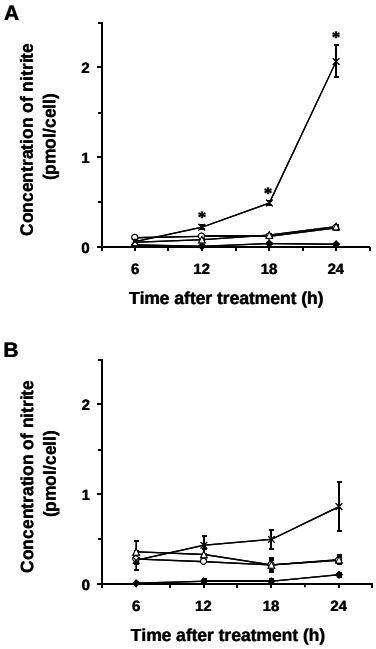

Fig. 4. Nitrite (NO_2^-) levels produced per viable cell in RAW 264.7 (A) and AMJ2-C11 (B) macrophage cultures at 6 h, 12 h, 18 h and 24 h after exposure to rIFN-γ (O), influenza virus (Δ) or a combination of influenza virus + rIFN-γ (×). The symbol of diamond (♦) represents controls (untreated cells). Values are means ± SEM of triplicates in a single experiment. $^*p < 0.05$ compared to rIFN-γ and influenza virus.

nificantly increased NO production in comparison with untreated cells ($p < 0.05$) in whose supernatants the determined NO_2^- levels were under the lower limit of detection except at 24 h for AMJ2-C11 cells. The maximum NO_2^- levels for both cell lines after rIFN-γ treatment were determined at 24 h: 0.78 ± 0.08 μmol/ml (for RAW 264.7 cells) and 0.59 ± 0.09 μmol/ml (for AMJ2-C11 cells). The NO_2^- levels produced per viable cell were similar to NO production as for NO_2^- concentrations determined in supernatants of RAW 264.7 macrophage cultures upon the treatment with rIFN-γ (Fig. 4A). A difference between NO_2^- levels produced per viable cell and NO_2^- concentrations determined in supernatants appeared with AMJ2-C11 macrophages, *i.e.* the NO_2^- levels produced per viable cell were highest at 6 h (0.28 ± 0.06 pmol/cell) and afterwards they slightly declined (Fig. 4B). rIFN-γ induced the greatest expression of iNOS mRNA at 6 h in RAW 264.7 macrophages (Fig. 5A and B). At 12 h, iNOS mRNA levels decreased approximately by 50% and remained at a lower but the same level for further time points. In contrast to RAW 264.7 cells, iNOS mRNA to rIFN-γ peaked at 24 h

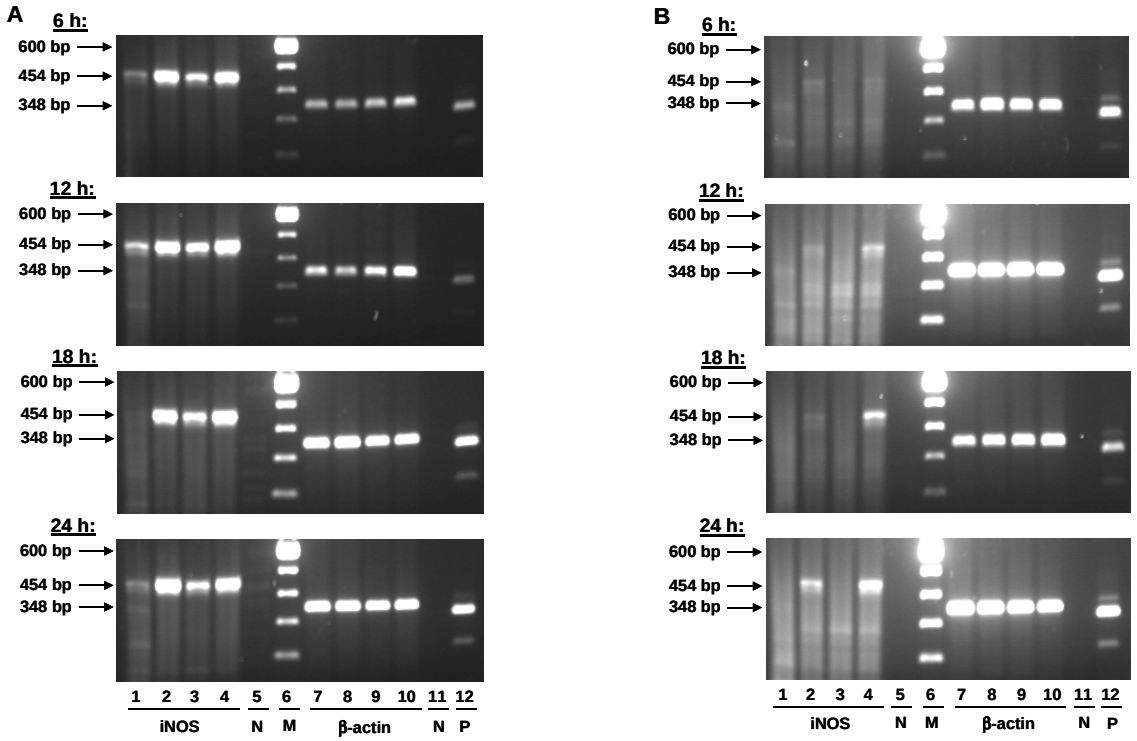

Fig. 5. Detection of iNOS mRNA expression by RT-PCR analysis in RAW 264.7 (A) and AMJ2-C11 (B) macrophages at 6 h, 12 h, 18 h and 24 h after exposure to rIFN-γ, influenza virus or a combination of influenza virus + rIFN-γ. Lane 1 – control (untreated cells); lane 2 – rIFN-γ; lane 3 – influenza virus; lane 4 – combination of influenza virus + rIFN-γ; lanes 7, 8, 9 and 10 indicate β-actin mRNA expression (internal control); lane 5 (N) – negative control for iNOS primers performed with nuclease-free water; lane 11 (N) – negative control for β-actin primers performed with nuclease-free water; lane 12 (P) – positive control performed with RNA from kit (323 bp); lane 6 (M) – molecular weight marker (100 bp DNA Ladder)

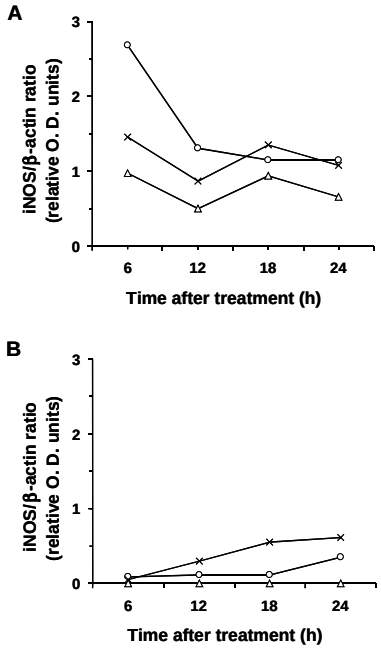

Fig. 6. Densitometric analysis of iNOS mRNA expression in RAW 264.7 (A) and AMJ2-C11 (B) macrophages at 6 h, 12 h, 18 h and 24 h after exposure to rIFN-γ (○), influenza virus (Δ) or a combination of influenza virus + rIFN-γ (×). For RAW 264.7 macrophages (A), each point represents a value subtracted from the value of control, and the curve of iNOS mRNA expression in control group of cells (untreated cells) is not presented. For AMJ2-C11 macrophages (B), the curve of iNOS mRNA expression in control group of cells (untreated cells) is not presented, since iNOS mRNA was not detected in this group of cells

post-exposure in AMJ2-C11 macrophages (Fig. 6A and B). Additionally, it should be noted that untreated RAW 264.7 macrophages expressed detectable levels of iNOS mRNA during the time course experiment. However, quantification of iNOS products by densitometry showed that iNOS mRNA levels in the control group of cells did not overlap iNOS mRNA levels either for the treatment with rIFN-γ or for the other treatments.

Effect of influenza virus on NO production and iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages

Treatment of RAW 264.7 macrophages with influenza virus resulted in a significant increase of NO synthesis compared to untreated cells at 12 h, 18 h and 24 h ($p < 0.05$). The threshold point of NO production was at 12 h, when NO_2^- levels in cell culture supernatants rose to 0.26 ± 0.05 μmol/ml, *i.e*, higher than the lower limit of NO_2^- detection (Fig. 3A). At 18 h, NO_2^- levels increased approximately 1.5-fold, and at 24 h, NO_2^- levels were 0.46 ± ± 0.04 μmol/ml. The conversion of NO_2^- concentrations determined in supernatants of RAW 264.7 macrophages into the NO_2^- levels produced per viable cell revealed a difference in expression of curves. The curve of NO_2^- concentrations in supernatants did not overlap either the curve for treatment with rIFN-γ or the curve for treatment with combination of influenza virus + rIFN-γ at any time point (Fig. 3A). The expression of NO_2^- levels produced per viable cell showed that the curve for the treatment with influenza virus overlapped the curve for the treatment with rIFN-γ at 18 h and 24 h (Fig. 4A). However, that was not significant ($p > > 0.05$). This discrepancy occurred because of the death of cells, whereas the cell viability upon the treatment with rIFN-γ was maintained over time (Fig. 1A). RT-PCR analysis revealed that RAW 264.7 macrophages treated with influenza virus already at 6 h expressed detectable levels of iNOS mRNA (Fig. 5A). At the latter time point, iNOS mRNA levels were the greatest according to densitometry results (Fig. 6A). Afterwards, they declined approximately by 50% at 12 h. More importantly, the expression of iNOS mRNA was prolonged for the further time points. However, iNOS mRNA levels in cells exposed to influenza virus were lower in comparison with rIFN-γ treated cells.

As shown in Fig. 3B, influenza virus increased NO_2^- levels in supernatants of AMJ2-C11 macrophage cultures. The highest concentration of NO_2^- was 0.47 ± 0.1 μmol/ml at 12 h. Afterwards, the NO_2^- concentrations decreased approximately twofold at 18 h and 24 h. The curve of NO_2^- formation in AMJ2-C11 macrophages for the treatment with influenza virus overlapped neither the curve for treatment with rIFN-γ nor the curve for the treatment with a combination of influenza virus + rIFN-γ except at 6 h (Fig. 3B). In terms of the manner of curve expression, NO_2^- concentrations determined in supernatants of AMJ2-C11 macrophage cultures showed the similarity with NO_2^- levels produced per viable cell (Fig. 3B and 4B). However, we did not detect iNOS mRNA expression at any time point after exposure of AMJ2-C11 macrophages to influenza virus (Fig. 5B and 6B). Noteworthy, our previous experiment carried out with AMJ2-C11 macrophages for 24 h revealed the capability of influenza virus to stimulate iNOS mRNA expression in these cells (Fig. 7D and E). Results of this experiment will be discussed in the context of the present study later in the paper.

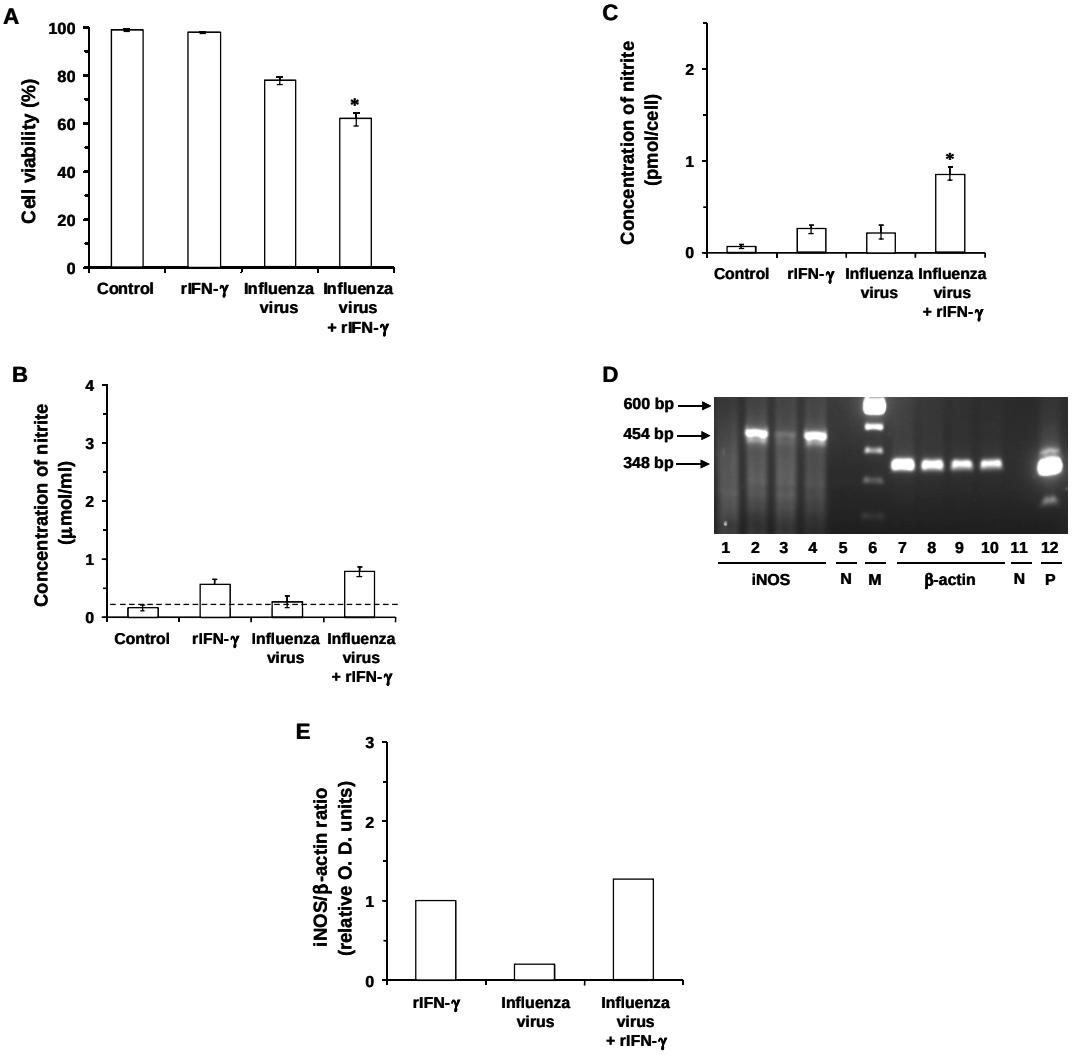

Fig. 7. Cell viability (A), nitrite (NO_2^-) concentrations in supernatants (B), NO_2– levels produced per viable cell (C), detection of iNOS mRNA by RT-PCR (D) and densitometric analysis of iNOS mRNA expression (E) in AMJ2-C11 macrophage cultures after 24 h exposure to rIFN-γ (1 ng/ml), influenza virus (10^6 $TCID_{50}$/ml) or a combination of influenza virus + rIFN-γ. For cell viability and NO_2– levels, the values are presented as means ± SEM of triplicates in a single experiment. $^*p < 0.05$ compared to rIFN-γ and influenza virus. In the graph B: the dashed line crosses Y axis at the point of 0.24 μmol/ml indicating the lower limit of NO_2^- detection. In the picture D: lane 1 – control (untreated cells); lane 2 – rIFN-γ; lane 3 – influenza virus; lane 4 – combination of influenza virus + rIFN-γ; lanes 7, 8, 9 and 10 indicate β-actin mRNA expression (internal control); lane 5 (N) – negative control for iNOS primers performed with nuclease-free water; lane 11 (N) – negative control for β-actin primers performed with nuclease-free water; lane 12 (P) – positive control performed with RNA from kit (323 bp); lane 6 (M) – molecular weight marker (100 bp DNA Ladder)

Effect of the combination of influenza virus + rIFN-γ on NO production and iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages

RAW 264.7 macrophages enhanced greatly NO synthesis in response to the treatment with combination of influenza virus + rIFN-γ compared to influenza virus or rIFN-γ (Fig. 3A). The measurement of NO_2^- levels in cell cultures revealed that a greater effect of the combination on NO production occurred at 18 h. This effect peaked at 24 h, when NO_2^- levels for exposure to the combination overlapped 6.5-fold and 3.8-fold NO_2^- levels for the treatments with influenza virus and rIFN-γ, respectively (Fig. 3A). The expression of NO_2– levels produced per viable cell showed a similar fashion of the curve of NO production as for NO_2^- levels determined in supernatants of RAW 264.7 macrophage cultures with a greater effect at 12 h, 18 h and 24 h (Fig. 4A). The combination of influenza virus + rIFN-γ stimulated expression of iNOS gene in RAW 264.7 macrophages at all time points as it was detected by RT-PCR analysis (Fig. 5A). However, the densitometry show-

ed that the gene expression was greater only at 18 h, when iNOS mRNA levels for treatment with the combination increased by 30% and 15% *versus* its mRNA levels for the treatments with influenza virus and rIFN-γ, respectively (Fig. 6A). At 6 h, 12 h and 24 h, the iNOS gene responded to the combination in higher mRNA levels compared to its mRNA levels upon the treatment with influenza virus, although they did not overlap iNOS mRNA levels for the exposure to rIFN-γ.

A different situation with the time course of NO production occurred in AMJ2-C11 macrophages treated with the combination of influenza virus + rIFN-γ. Challenge of macrophages with this combination rose NO_2^- formation in cell cultures, resulting in its maximum levels at 12 h: 0.49 ± 0.01 μmol/ml (Fig. 3B). As distinct from RAW 264.7 macrophages, NO_2^- concentrations in AMJ2-C11 macrophage cultures declined at 18 h and 24 h *versus* the concentrations at a previous time point (Fig. 3A and B). However, the expression of NO_2^- levels produced per viable cell revealed a greater effect of the combination of influenza virus + rIFN-γ on NO production in AMJ2-C11 macrophages compared to either component alone at 18 h and 24 h (Fig. 4B). At the latter time point, NO_2^- levels for the combination increased more than threefold *versus* NO_2^- levels for both influenza virus and rIFN-γ treatments. It should be noted that this augmentation of NO_2^- levels did not have a statistical significance ($p > 0.05$). In AMJ2-C11 macrophages, the combination of influenza virus + rIFN-γ stimulated expression of iNOS gene greater than with exposure to rIFN-γ at 12 h, 18 h and 24 h (Fig. 5B). Densitometric analysis showed that iNOS mRNA levels for the combination were increasing gradually over time, and they peaked at 24 h overlapping iNOS mRNA levels for the treatment with rIFN-γ by more than 40% (Fig. 6B). Overall, iNOS mRNA levels in AMJ2-C11 macrophages correlated with NO_2^- levels produced per viable cell upon this treatment.

DISCUSSION

This study demonstrated influenza virus ability to stimulate iNOS gene expression in RAW 264.7 cells and in the presence of rIFN-γ synergistically increase NO production in both RAW 264.7 and AMJ2-C11 macrophage cell lines. iNOS gene induction by influenza virus differed by cell line. AMJ2-C11 macrophages incubated with influenza virus for 24 h resulted in dramatically reduced cell viability and increased NO synthesis that was mediated through iNOS gene induction (Fig. 7). Furthermore, influenza virus in combination with rIFN-γ stimulated iNOS mRNA expression more than by either component alone, resulting in a synergistic effect. In terms of NO production per viable cell, this combination enhanced NO_2^- levels in cell cultures approximately fourfold and 3.3-fold as compared to with NO_2^- levels for the treatments with influenza virus and rIFN-γ, respectively. The latter result is statistically significant ($p < 0.05$), and it indicates a synergistic effect of the combination on NO production in AMJ2-C11 macrophages. However, in the present time course experiment iNOS mRNA expression was not detected in AMJ2-C11 macrophages, despite the determination of NO_2^- formation in cell cultures. We relate this discrepancy to the fact that iNOS mRNA contains multiple motifs of the same nucleotide order (AUUUA) in the 3′ untranslated region, which are responsible for mRNA destabilization and consequently for the short lifespan of mRNA (20). Noteworthy, depending on the origin of stimulus and the type of cell, the half-life of iNOS mRNA varies; *e.g.*, in J774 macrophage cell line treated with LPS it is between 5 h and 6 h (21). Hence, instability and a rapid degradation of iNOS mRNA are the possible reasons why during the time course experiment we did not detect iNOS mRNA expression in AMJ2-C11 macrophages upon the treatment with influenza virus at the earliest 6 h time point. Furthermore, nitrite, being a stable and predominant metabolite of NO in aqueous solutions, accumulates in the medium of cell cultures (22). That explains the detectable NO_2– levels in AMJ2-C11 macrophage cultures after exposure to influenza virus at 6 h, 12 h, 18 h and 24 h, while iNOS mRNA is already degraded resulting in the undetectable levels. On the other hand, in the previous experiment, AMJ2-C11 macrophages responded to influenza virus in a weak iNOS gene expression at 24 h, and iNOS mRNA levels were very low comparing with the treatments for rIFN-γ or the combination of influenza virus + rIFN-γ (Fig. 7D and E). Several other experiments with AMJ2-C11 macrophages in the presence of 1% heat-inactivated FBS demonstrated a strong iNOS mRNA expression in response to influenza virus as well as to rIFN-γ or the combination of both at 24 h incubation (data not shown). It indicates a capacity of FBS (or its biologically active constituents) to stabilize iNOS mRNA and to prolong its expression in macrophages.

The finding that influenza virus can stimulate iNOS mRNA expression and enhance NO synthesis in RAW 264.7 and AMJ2-C11 cells suggests that the influenza virus is replicating within macrophages. The reduced density (and survival) of macrophages in our experiments could be the outcome of viral replication in the macrophage. It is known that

the influenza virus enters and infects murine macrophages through the binding to mannose receptors (23). Pahl & Baeuerle (24) demonstrated that the influenza virus hemagglutinin (HA) can mediate the activation of a cellular transcription factor, the nuclear factor kappa B (NF-κB). Since the promoter regions I and II of the murine macrophage iNOS gene contains NF-κB binding sites (25), it is plausible that HA synthesis and intracellular accumulation due to the replication of influenza virus in macrophages leads to the induction of iNOS gene expression. Further, the region II of iNOS gene promoter contains also IFN-stimulated response elements (ISRE) for the interferon regulatory factor-1 (IRF-1) for which expression is inducible by IFN-γ (7, 25). NF-κB and IRF-1 binding motifs are in tandem and located in the iNOS promoter region II. In this situation, the binding elements in the gene promoter are found to be responsible for synergy between transcription factors (NF-κB and IRF-1) in the stimulation of other genes, for instance, the vascular cell adhesion molecule 1 (VCAM-1) gene (26). The synergistic effect between LPS (via the activation of NF-κB) and IFN-γ on induction of iNOS gene and increase of NO production in macrophages has been well demonstrated in a number of studies (7). It is noteworthy that recently Korhonen et al. (21) showed the capability of IFN-γ to mediate stabilization of iNOS mRNA and to prolong its half-life considerably in the combination with LPS. Finally, a study of Paludan et al. (27) demonstrated that HSV-2 synergistically enhanced NO synthesis in murine macrophages in the presence of IFN-γ, and it was mediated through the activation of NF-κB. Taken together with the present data, it can be explained why the combination of influenza virus + rIFN-γ synergistically increases NO production in RAW 264.7 and AMJ2-C11 macrophages.

It should be noted that upon treatment with the combination of influenza virus + rIFN-γ, RAW 264.7 macrophages produced substantially more NO than AMJ2-C11 macrophages during the time course experiment. However, in terms of iNOS mRNA expression in RAW 264.7 cells, this combination was not synergistically discrepant from that observed in AMJ2-C11 cells. The difference can be explained by the regulation of the iNOS gene at both post-transcriptional and transcriptional levels (7). We propose that increasing the amount of NO in RAW 264.7 macrophage cultures exerted a negative autoregulatory feedback on iNOS mRNA expression, while relatively low levels of NO produced in AMJ2-C11 macrophage cultures did not have the same effect on iNOS mRNA expression (28). Thus, also on the basis of recent report by Akaike et al. (29), it is most likely that this occurrence is related to NO capacity to induce nitration of the nucleotide base guanosine and formation of the 8-nitroguanosine in RNA structure resulting in the inhibition of RNA function. Furthermore, the expression of NO_2^- levels produced per viable cell revealed that a less number of cells upon the treatment with the combination produced more NO than a greater number of cells upon the treatments with either the influenza virus or rIFN-γ in both RAW 264.7 and AMJ2-C11 macrophage cultures.

Assuming that the basal levels of IFN-γ are produced *in vivo* constitutively, we were using the lowest concentration of rIFN-γ (1 ng/ml) in experiments. In this way, we tried to imitate the natural conditions *in vivo* during influenza virus infection as much as possible. The results of our experiments clearly demonstrate that the influenza virus in combination with IFN-γ is the essential condition for triggering the high-output generation of NO in monocytes/macrophages (RAW 264.7 cells) and lung alveolar macrophages (AMJ2-C11 cells). Moreover, this combination reduces cell viability more than either component alone. It is an indication that IFN-γ does not appear to be cytotoxic independently of influenza virus. Acting both together, these factors enhance greatly production and yield of NO, which, in turn, exerts the cytotoxic effect. The cytotoxicity of NO is mediated via reactive nitrogen oxides (*e.g.*, peroxynitrite) or NO-induced nitration of cellular RNAs, and its implication in the pathogenesis of injury of lung tissues during viral infections was well recognized in mouse model experiments (10, 11, 29). These experiments *in vivo* showed that treatment of virus-infected mice with N^{ω}-monomethyl-L-arginine and N^{G}-monomethyl-1-arginine, the non-selective inhibitors of NOS, did not reduce pulmonary viral titers, but considerably improved the survival rate and lung compliance of mice compared with placebo. Strong pulmonary inflammation is a characteristic feature of the immune response to primary influenza virus infection, which also includes recruitment of macrophages into lungs. An origin and kinetics of pulmonary macrophages during acute inflammation were demonstrated in a study performed by Blussé van Oud Alblas et al. (30). After exposure of mice to aerosolized heat-killed bacillus Calmette-Guérin (BCG), they found that the number of pulmonary macrophages rose very rapidly in the first 24 h after exposure due to migration of monocytes from blood into lung tissues, and this was sevenfold higher than the normal monocyte influx in unexposed mice over the same period of time. Among other immune cells, macrophages play one of the major roles in exacerbation of the inflamma-

tion. In this respect, our findings provide more insights into the contribution of macrophages in the lung damage during influenza virus infection and perhaps relate to difference in influenza morbidity and mortality in "high-risk" populations.

In accordance with the presented results, we conclude that the influenza virus possesses the capacity to mediate iNOS gene induction in macrophages and in combination with IFN-γ synergistically increases NO synthesis in these cells. From the practical viewpoint, this is important for understanding the mechanism by which the influenza virus is implicated in the development of pulmonary complications, specifically in the elderly population.

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References

1. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001; 357: 593–615.
2. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992; 256: 225–8.
3. Gross SS, Levi R. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J Biol Chem 1992; 267: 25722–9.
4. Nicholson S, Bonecini-Almeida M da G, Lape e Silva JR, Nathan C, Xie QW, Mumford R et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J Exp Med 1996; 183: 2293–302.
5. Sharara AI, Perkins DJ, Misukonis MA, Chan SU, Dominitz JA, Weinberg JB. Interferon (IFN)-α activation of human blood mononuclear cells in vitro and in vivo for nitric oxide synthase (NOS) type 2 mRNA and protein expression: possible relationship of induced NOS2 to anti-hepatitis C effects of IFN-α *in vivo*. J Exp Med 1997; 186: 1495–502.
6. Asano K, Chee CBE, Gaston B, Lilly CM, Gerard C, Drazen JM et al. Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. Proc Natl Acad Sci USA 1994; 91: 10089–93.
7. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997; 15: 323–50.
8. Rimmelzwaan GF, Baars MMJW, Lijster P, Fouchier RAM, Osterhaus ADME. Inhibition of influenza virus replication by nitric oxide. J Virol 1999; 73: 8880–3.
9. Reiss CS, Komatsu T. Does nitric oxide play a critical role in viral infections? J Virol 1998; 72: 4547–51.
10. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM et al. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci USA 1996; 93: 2448–53.
11. Adler H, Beland JL, Del-Pan NC, Kobzik L, Brewer JP, Martin TR et al. Suppression of herpes simplex virus type 1 (HSV-1)-induced pneumonia in mice by inhibition of inducible nitric oxide synthase (iNOS, NOS2). J Exp Med 1997; 185: 1533–40.
12. Fultz MJ, Barber SA, Dieffenbach CW, Vogel SN. Induction of IFN-γ in macrophages by lipopolysaccharide. Int Immunol 1993; 5: 1383–92.
13. Di Marzio P, Puddu P, Conti L, Belardelli F, Gessani S. Interferon g upregulates its own gene expression in mouse peritoneal macrophages. J Exp Med 1994; 179: 1731–6.
14. Carvalho-Pinto CE, Garcia MI, Mellado M, Rodriguez-Frade JM, Martin-Caballero J, Flores J et al. Autocrine production of IFN-γ by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/*lpr* mice. J Immunol 2002; 169: 1058–67.
15. Lennette EH, Lennette DA, Lennette ET. Diagnostic procedures for viral, rickettsial, and chlamydial infections. Washington, DC: American Public Health Association 1995.
16. Kärber G. Beitrang zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exptl Path Pharmakol 1931; 162: 480–3.
17. Thompson WR. The use of moving averages and interpolation to estimate median effective dose. Bacteriol Rev 1947; 11: 115–47.
18. Han B, DuBois DC, Boje KMK, Free SJ, Almon RR. Quantification of iNOS mRNA with reverse transcription polymerase chain reaction directly from cell lysates. Nitric Oxide: Biol Chem 1999; 3: 281–91.
19. Zhu H, Naito M, Umezu H, Moriyama H, Takatsuka H, Takahashi K et al. Macrophage differentiation and expression of macrophage colony-stimulating factor in murine milky spots and omentum after macrophage elimination. J Leukoc Biol 1997; 61: 436–44.
20. Hattori Y, Gross SS. Cycloheximide induces nitric oxide syhthase mRNA in vascular smooth muscle cells by prolonging mRNA lifetime. Biochem Mol Biol Int 1995; 37: 439–45.
21. Korhonen R, Lahti A, Hämäläinen M, Kankaanranta H, Moilanen E. Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Mol Pharmacol 2002; 62: 698–704.
22. Fukuto JM. Chemistry of nitric oxide: biologically relevant aspects. Adv Pharmacol 1995; 34: 1–15.

23. Reading PC, Miller JL, Anders EM. Involvement of the mannose receptor in infection of macrophages by influenza virus. J Virol 2000; 74: 5190–7.
24. Pahl HL, Baeuerle PA. Expression of influenza virus hemagglutinin activates transcription factor NF-kB. J Virol 1995; 69: 1480–4.
25. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW et al. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. Proc Natl Acad Sci USA 1993; 90: 9730–4.
26. Neish AS, Read MA, Thanos D, Pine R, Maniatis T, Collins T. Endothelial interferon regulatory factor 1 cooperates with NF-κB as a transcriptional activator of vascular cell adhesion molecule 1. Mol Cell Biol 1995; 15: 2558–69.
27. Paludan SR, Ellermann-Eriksen S, Mogensen SC. NF-κB activation is responsible for the synergistic effect of herpes simplex virus type 2 infection on interferon-γ-induced nitric oxide production in macrophages. J Gen Virol 1998; 79: 2785–93.
28. Hinz B, Brune K, Pahl A. Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages. Biochem Biophys Res Commun 2000; 271: 353–7.
29. Akaike T, Okamoto S, Sawa T, Yoshitake J, Tamura F, Ichimori K et al. 8-Nitroguanosine formation in viral pneumonia and its implication for pathogenesis. Proc Natl Acad Sci USA 2003; 100: 685–90.
30. Blussé van Oud Alblas A, van der Linden-Schrever B, van Furth R. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intra-alveolar administration of aerosolized heat-killed BCG. Am Rev Respir Dis 1983; 128: 276–81.

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GRIPO VIRUSAS IR GAMA INTERFERONAS SINERGETIŠKAI PADIDINA AZOTO MONOKSIDO GAMYBĄ RAW 264.7 IR AMJ2-C11 MAKROFAGUOSE

S a n t r a u k a

Laisvieji radikalai, įskaitant azoto monoksidą (NO), pažeidžia plaučius gripo infekcijos metu. Makrofagai yra vienos iš pagrindinių ląstelių, gausiai gaminančių NO kaip atsaką į gama interferoną (IFN-γ) – citokiną, sintezuojamą šios virusinės infekcijos metu. Tačiau kaip gripo virusas sukelia didelių NO kiekių gamybą makrofaguose, lieka neaišku. Mes iškėlėme hipotezę, kad gripo viruso infekcija, sąveikaudama su IFN-γ, sinergetiškai padidina NO produkciją makrofaguose. Siekiant tai ištirti, RAW 264,7 ir AMJ2-C11 pelės makrofagų ląstelių linijos buvo paveiktos IFN-γ, gripo A/PR/8/34 (H1N1) virusu arba jų abiejų kombinacija. Tyrimo kontrolei pasirinktos nepaveiktos ląstelės. Praėjus 6, 12, 18 ir 24 valandoms buvo įvertintas ląstelių gyvybingumas, išmatuoti nitritų lygiai makrofagų kultūrose; ląstelės buvo surinktos siekiant išskirti RNR bei nustatyti indukuojamos NO sintetazės (iNOS) iRNR. Rezultatai atskleidė, kad gripo virusas nulėmė iNOS geno indukciją RAW 264.7 makrofaguose ir, veikdamas kartu su IFN-γ, sinergetiškai padidino NO sintezę abiejose ląstelių linijose. Be to, minėta kombinacija labiau sumažino ląstelių gyvybingumą negu kiekvienas komponentas atskirai paėmus. Šie duomenys yra svarbūs išaiškinant tas aplinkybes, kurioms esant gripo virusas sukelia perteklinę NO gamybą makrofaguose, galinčią nulemti sunkų plaučių uždegimą.

Raktažodžiai: gripo virusas, gama interferonas, azoto monoksidas, makrofagai