


CD38 modulates respiratory syncytial virus-driven proinflammatory processes in human monocyte-derived dendritic cells

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Introduction

Human respiratory syncytial virus (RSV) is a negative single-stranded RNA virus of the *Pneumoviridae* family, previously classified in the *Paramyxoviridae* family.¹ RSV is the commonest cause of lower respiratory tract infection (LRTI) in children and the major cause of infantile bronchiolitis: up to 70% of newborn infants become infected during their first year of life, and virtually all children by 2 years of age have been infected by RSV.² In most cases the infection does not cause serious damage; however, some individuals, such as preterm infants, are at

Summary

Respiratory syncytial virus (RSV) is the most common cause of hospitalization due to bronchiolitis in infants. Although the mechanisms behind this association are not completely elucidated, they appear to involve an excessive immune response causing lung pathology. Understanding the host response to RSV infection may help in the identification of targets for therapeutic intervention. We infected *in-vitro* human monocyte-derived dendritic cells (DCs) with RSV and analysed various aspects of the cellular response. We found that RSV induces in DCs the expression of CD38, an ectoenzyme that catalyses the synthesis of cyclic ADPR (cADPR). Remarkably, CD38 was under the transcriptional control of RSV-induced type I interferon (IFN). CD38 and a set of IFN-stimulated genes (ISGs) were inhibited by the anti-oxidant N-acetyl cysteine. When CD38-generated cADPR was restrained by 8-Br-cADPR or kuromanin, a flavonoid known to inhibit CD38 enzymatic activity, RSV-induced type I/III IFNs and ISGs were markedly reduced. Taken together, these results suggest a key role of CD38 in the regulation of anti-viral responses. Inhibition of CD38 enzymatic activity may represent an encouraging approach to reduce RSV-induced hyperinflammation and a novel therapeutic option to treat bronchiolitis.

Keywords: inflammation; interferons; monocyte-derived dendritic cells; respiratory syncytial virus.

higher risk for developing severe bronchiolitis with viral spreading to the lower respiratory tract. High morbidity, high hospitalization rates and significant mortality rates are features of RSV-associated disease.³ Multiple epidemiological studies have clearly demonstrated that RSV bronchiolitis is frequently associated with subsequent persistent wheezing, childhood asthma or both.^{4,5} Neither a safe vaccine nor effective therapy are yet available.^{6,7}

The causes of severe RSV disease are not fully understood, but might involve both magnitude and quality of the host immune response. RSV infection initiates a cascade of events leading to the recruitment and activation

Abbreviations: 8-Br-cADPR, 8-Bromo-cADP ribose; cADPR, cyclic adenosine diphosphate ribose; IFN, interferon; ISGs, IFN-stimulated genes; LRTI, lower respiratory tract infection; MDDC, monocyte-derived dendritic cells; NAC, N-acetyl-cysteine; NAD⁺, nicotinamide adenine dinucleotide; RSV, respiratory syncytial virus; SOD2, superoxide dismutase 2

of immune effectors, including monocyte/macrophages and dendritic cells (DCs) which, in turn, trigger the production of cytokines and chemokines, which orchestrate and potentiate the immune response.⁸ However, when exacerbated, the immune response to RSV may lead to tissue damage and to the establishment of bronchiolitis.⁹

In this scenario, a crucial role is played by DCs that act as a portal for virus invasion as well as potent antigen-presenting cells, positioned to link innate to adaptive immune responses.¹⁰ Indeed, some studies suggest that myeloid DCs, along with macrophages, are the major source of type I interferons (IFNs) during pulmonary viral infections.¹¹ There is also evidence that DCs, along with epithelial cells, produce IFN lambda (i.e. type III IFN) in response to RSV.¹² The induction of types I and III IFNs, of IFN-stimulated genes (ISGs) and of proinflammatory cytokines and chemokines are hallmarks of anti-RSV immunity.^{13,14} It has been shown that type I IFN production in response to RSV infection not only induces an anti-viral state, but also operates to amplify proinflammatory responses in the respiratory tract. This process is a natural component of the early inflammatory response to RSV infection, designed to keep the virus under control until adaptive immunity is activated to clear the virus.¹⁵ However, type I IFN activation must be tightly regulated in order to reduce viral load but not allow excessive inflammatory cytokine and chemokine production. It is possible to hypothesize that the onset of RSV-induced bronchiolitis in some human patients is related to genetically or environmentally driven alterations to this balance.¹⁵

As well as the inflammatory response, RSV induces oxidative stress, defined as a disruption of the pro-oxidant/antioxidant balance,¹⁶ with cell death, loss of immune function, increased viral replication and inflammatory response, thereby contributing to pathogenesis.

In a previous study, we have shown that RSV triggers type I IFNs expression in monocyte-derived DCs (MDDCs).¹⁷ We also showed that RSV infection induces up-regulation of surface CD38, a marker of DCs activation induced by multiple inflammatory stimuli.¹⁸ CD38 is a multifunctional ectoenzyme that catalyses the synthesis of adenosine diphosphate ribose (ADPR) and cyclic ADPR (cADPR), second messengers involved in the regulation of cytoplasmic Ca²⁺ fluxes.^{19–21} CD38 also influences both innate and adaptive immune responses by regulating the trafficking of cells (e.g. neutrophils, DCs) to the sites of inflammation²² and multiple aspects related to DCs maturation, such as chemotaxis and transendothelial migration, longevity, interleukin (IL)-12 production and T helper type 1 (Th1) polarization.¹⁸

Here, we investigated the role of CD38 in the regulation of cellular responses to RSV in infected MDDCs with the aim of identifying possible lines of clinical intervention to ameliorate RSV-associated LRTI treatment.

Materials and methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Blood donors provided written informed consent.

Chemicals

8-Br-cADPR was from Santa Cruz Biotechnology (Dallas, TX); nicotinamide adenine dinucleotide (NAD)⁺, potassium dihydrogen phosphate (KH₂PO₄), acetonitrile [high-performance liquid chromatography (HPLC)-grade reagent], kuromanin, N-acetylcysteine (NAC), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; adenosine deaminase inhibitor), dypiridamole (phosphodiesterase inhibitor) and levamisole (alkaline phosphatase inhibitor) were all from Sigma-Aldrich (St Louis, MO). All the chemical reagents used were of analytical grade.

Purification and culture of MDDCs

Human monocytes were purified from peripheral blood of healthy blood donors (courtesy of Dr Girelli, 'Centro Trasfusionale Policlinico Umberto I', University La Sapienza, Rome, Italy) using Ficoll gradients (lympholyte-H; Cedarlane, Burlington, ON). CD14 cells were purified by anti-CD14 monoclonal antibody (mAb)-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were cultured in the presence of human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (50 ng/ml each; Immunological Sciences, Rome, Italy); after 6 days immature MDDCs were washed and analysed using cytofluorimetry for the expression of the surface markers CD11c, CD14, CD83 and CD38 employing the fluorochrome-conjugated mAbs (BD Biosciences, San Jose, CA). MDDCs were used in the experiments if > 90% CD11c and < 5% CD14. All the experiments were performed with MDDCs obtained from different donors.¹⁷

Preparation of RSV stocks and MDDCs infection

Plaque-purified human RSV [type A2 strain from the American Type Culture Collection (ATCC)] was grown in human epithelial type 2 (HEp-2) cells.²³ Mock-infected HEp-2 cells were similarly processed to generate the mock control stock preparation. Viral titres were determined by plaque assay.

MDDCs (10⁶ cell/ml) were incubated with RSV at multiplicity of infection (MOI) equal to 1. After 2 hr at 37°C cells were extensively washed and incubated in 1 ml of complete medium at 37°C, 5% CO₂ for 20 hr post-

infection (p.i.). As negative control, an equal volume of mock control medium was added to MDDCs.

In some experiments, cells were pretreated for 20 min with kuromanin (100 μ M), 8-Br-cADPR (5 μ g/ml), NAC (2 mM) or a combination of human anti-human IFN (IFN)- α and IFN- β antibody (National Institutes of Health, Bethesda, ND). The inhibitors were re-added at the same concentration to the culture medium after the infection and the washing steps.

Apoptosis assay

Apoptosis was measured in RSV-infected MDDCs after 20 hr in the presence of NAC or 8 Br-cADPR using an annexin V apoptosis detection kit (BD Biosciences). The assay was carried out following the manufacturer's protocol. Briefly, infected cells were incubated with different dilutions of NAC or 8-Br-cADPR for 20 hr. Cells were harvested, washed in phosphate-buffered saline (PBS), pelleted and resuspended in binding buffer; 100 μ l of the cell suspension (10^5 cells) was then mixed with 5 μ l of fluorescein isothiocyanate (FITC) annexin V and 5 μ l propidium iodide (PI). The samples were kept in the dark and incubated for 15 min at room temperature prior to the addition of binding buffer. Samples were analysed by cytofluorimetry to detect apoptotic cells and dead cells.

TaqMan-based real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted at 4 and 20 hr p.i. from RSV-infected and mock-infected MDDCs using the total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON) and was retrotranscribed as previously described.¹⁷ Quantitative real-time PCR was carried out with a Viiia7 real-time PCR system (Applied Biosystems, Foster City, CA). Primers and probes for each gene were added to the Probes Master Mix at 500 and 250 nM, respectively, in a final volume of 20 μ l.^{17, 24} The housekeeping gene β -glucuronidase (or human β -actin) was used as internal control. All the determinations were performed in duplicate. Gene expression values were calculated by the comparative Ct ($\Delta\Delta$ CT) method. Fold changes in gene expression levels were calculated by comparison with the gene expression in mock samples, which were assigned an arbitrary value of 1.

RSV amplification by RT-PCR

Total RNA was extracted from RSV-infected and mock-infected MDDCs and reverse transcription was carried out as described by Loebbermann and colleagues²⁵ using 1 μ g of total RNA for cDNA synthesis. Quantitative PCR (qPCR) specific for the RSV L gene was performed using 900 nM forward primer (5'-GAACTCAGTGTAGGTAGAA TGTTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTAT

CATTTTCTCTGCCAAT-3') and 175 nM probe (5'-6-carboxyfluorescein-TTTGAACCTGTCTGAACATTCCCGGT T-6-carboxytetramethylrhodamine-3'). Copy numbers were determined from standard curves of pCDNA3 containing a fragment of the RSV L-gene.

Adenosine generation assay

MDDCs were infected with RSV in the presence or absence of kuromanin, as described above. After the infection, cells (1×10^6 /ml) were suspended in AIM V serum-free medium (Invitrogen, Carlsbad, CA) containing a mix of EHNA, dipyrindamole and levamisole and incubated for 15 min at 37°C, 5% CO₂. NAD⁺ (100 M) was then added and cells were incubated for 30 min at 37°C. Following centrifugation (700 g; 5 min at 4°C) supernatants were collected in tubes containing 1 ml of ice-cold acetonitrile to stabilize adenosine. Supernatants were evaporated by speed-vacuum, reconstituted in mobile-phase buffer, and assayed by HPLC.

Chromatographic analysis was performed with an HPLC system (Beckman Gold 126/166NM; Beckman Coulter, Brea, CA) equipped with a reverse-phase column (Hamilton C18, 5 μ m; 250 \times 4.5 mm). Separation of nucleotides and nucleosides was performed using a mobile-phase buffer (0.125 M citric acid and 0.025 M KH₂PO₄), pH 5.1 with 8% acetonitrile for 10 min at a flow rate of 0.8 ml/min. UV absorption spectra were measured at 254 nm. HPLC-grade standards used to calibrate the signals were dissolved in AIM V serum-free medium, pH 7.4, 0.2 μ m sterile-filtered and injected in a buffer volume of 20 μ l.

The retention time (R_t , in min) of standard ADPR was: 3.2. Peak integration was performed using KARAT software (Beckman Coulter). Quantitative measurements were inferred by comparing the peak area of samples with calibration curve for peak area of standard compound.

Statistical analysis

All results are expressed as mean \pm standard error of the mean (SEM). Statistical significance was determined using a two-tailed, unpaired Student's *t*-test (* P < 0.05, ** P < 0.01). Values of P < 0.05 and P < 0.01 were considered significant (PRISM software; Graph-Pad Software Inc., San Diego, CA).

Results

CD38 expression is regulated by type-I IFNs in RSV-infected MDDCs

In a previous study we showed that RSV infection induces a significant up-regulation of CD38 surface expression as well

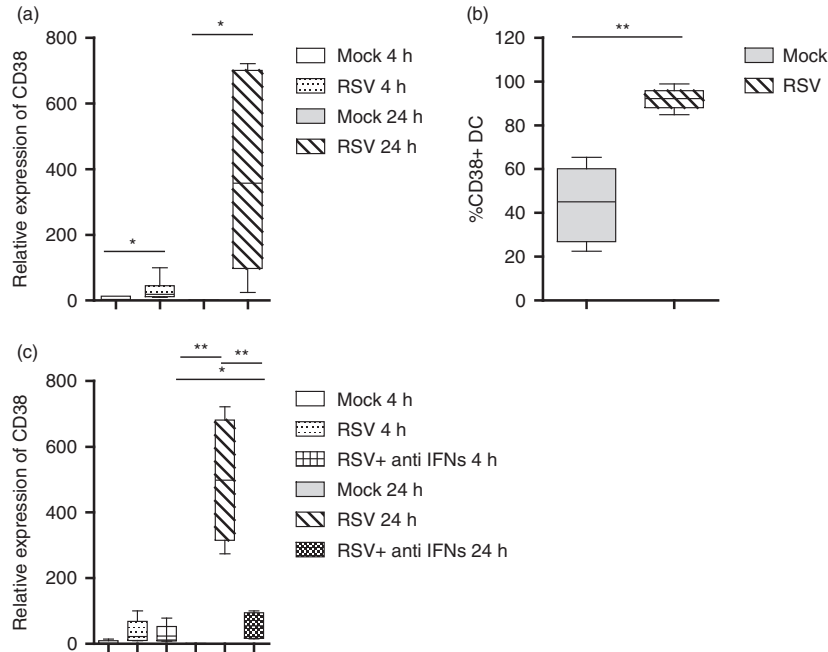


Figure 1. Respiratory syncytial virus (RSV)-induced CD38 expression in monocyte-derived dendritic cells (MDDCs). MDDCs were infected with RSV [multiplicity of infection (MOI) of 1] and in (c), cells were also treated with a combination of human anti-human interferon (IFN)- α and - β antibody. Mock-infected cells were added as control. In (a) and (c), total RNA was extracted at the indicated time-points, and the kinetics of mRNA expression for CD38 was evaluated by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR). The mRNA transcripts were normalized with respect to the endogenous reference (human β -actin) sample. Data were expressed as fold increase versus mock-treated cells at 4 hr [mean \pm standard error of the mean (SEM) of seven or five experiments in (a) and (c), respectively]. (b) RSV-infected MDDCs, after 20 hr, were analysed for the CD38 surface marker. Fluorescence data are as percentage of positive cells. Values are expressed as mean \pm SEM of five independent experiments. For all, statistical significance was determined using a two-tailed, unpaired Student's *t*-test (* P < 0.05, ** P < 0.01).

as IFN- β production in MDDCs.¹⁷ Here, we first analysed whether the increased expression of CD38 at the cell surface is paralleled by increased transcription of the CD38 gene. Infection of MDDCs by RSV was checked by measuring RSV L gene transcripts, as an index of viral copies (data not shown). As shown in Fig. 1a, RSV induced a significant increase of CD38 transcription compared to mock treated cells, mostly at 20 hr p.i. The levels of CD38 surface expression, checked in parallel by flow cytometry, confirmed the gene expression data (Fig. 1b). As type I IFNs activate CD38 expression,²⁶ we then asked whether the observed activation of CD38 was dependent upon type I IFNs produced by MDDCs in response to RSV infection. With this aim, we performed *in-vitro* infection experiments in the presence of a combination of neutralizing antibodies directed against type I IFNs (e.g. IFN- α and - β). As shown in Fig. 1c, blocking of type I IFNs signalling significantly reduced RSV-induced CD38 gene expression at 20 hr p.i., confirming our hypothesis.

CD38 and IFNs-dependent response induced by RSV are linked to oxidative stress

CD38 has been described to play a role in the regulation of oxidative stress and accumulation of reactive oxygen

species in eukaryotic cells,^{27,28} a process that is also a hallmark of RSV infection.²⁶ The observation that, in airway epithelial cells, RSV infection modulates the oxidative balance through up-regulation of superoxide dismutase 2 (SOD2),²⁹ prompted us to investigate whether the same event occurs in MDDCs. A strong induction of SOD2 expression was recorded in RSV-infected MDDCs, at either 4 or 20 hr p.i. Remarkably, in the presence of NAC, SOD2 gene expression decreased, reaching a significant reduction at 20 hr p.i. (Fig. 2a).

We measured the expression of an array of IFNs and ISGs that are key markers of the anti-RSV innate immune response, such as IFN- β , IFN- λ 1/IL-29, chemokine (C-C motif) ligand 5/regulated on activation, normal T cell expressed and secreted (CCL5/RANTES), myxovirus resistance A (MxA) and IFN-stimulated gene 15 (ISG15)^{26,30–33} in MDDCs infected in the presence or absence of NAC. As shown in Fig. 2b, all the genes analysed were up-regulated in infected MDDCs; in particular we noticed an early expression of IFN- β , while IFN- λ 1 and ISGs peaked at a later time-point p.i., suggesting two separate waves of transcription.

We found that NAC statistically reduced IFN- β induction at 4 and 20 hr p.i., while the expression of all ISGs was reduced at the later time-point in RSV-infected

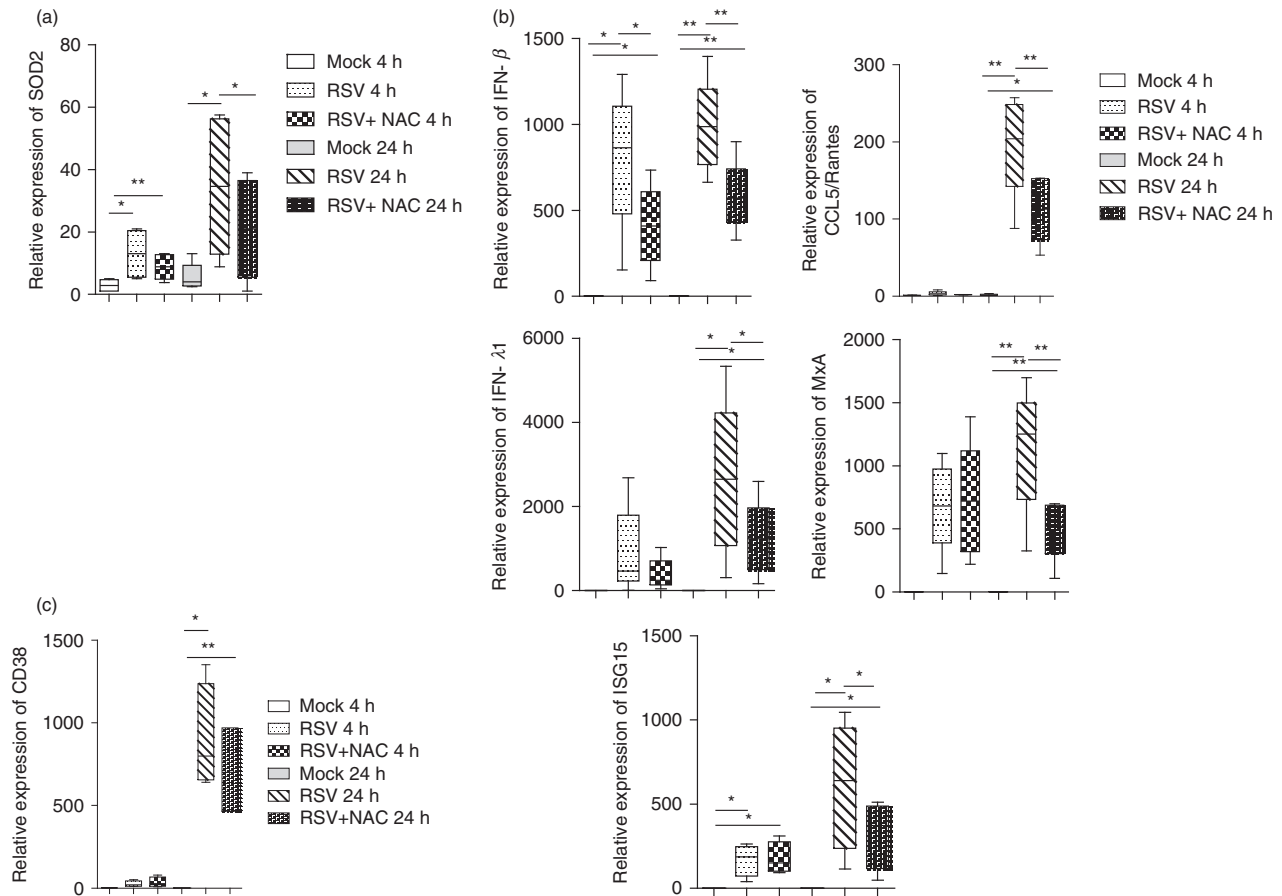


Figure 2. NAC treatment and genes expression in respiratory syncytial virus (RSV)-infected monocyte-derived dendritic cells (MDDCs). Cells were infected with RSV [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, some samples were pretreated with N-acetyl-cysteine (NAC) (2 mM) for 20 min. Mock-infected cells were added as control. After infection, cells were washed, incubated in fresh medium and, where indicated, NAC was re-added. At the indicated time-points, total RNA was extracted and kinetics of mRNA expression for all genes analysed was evaluated by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR). The mRNA transcripts were normalized with respect to the endogenous reference sample. Data were expressed as fold increase versus mock-treated cells at 4 hr [mean \pm standard error of the mean (SEM) of five experiments in (a) and (b), mean \pm standard error of the mean (SEM) of four experiments in (c)]. Statistical significance was determined using a two-tailed, unpaired Student's *t*-test (**P* < 0.05, ***P* < 0.01).

MDDCs. Finally, down-regulation of CD38 expression was recorded in NAC-treated MDCCs at 20 hr p.i. (Fig. 2c). The fact that ISGs are modulated by RSV and NAC is proof of IFN protein expression. Such evidence is important, as mRNA levels may not necessarily be correlated with the protein levels.

A cADPR antagonist inhibits RSV-driven proinflammatory responses in human MDDCs

Once established that either oxidative stress or type-I IFN responses drive the up-regulation of CD38 in RSV-infected MDDCs, we sought to determine whether the enzymatic functions of CD38 are involved in the activation of anti-viral and proinflammatory responses.

To this aim, MDDCs were infected with RSV in the presence of 8-Bromo-cADP ribose (8-Br-cADPR), a cell-

permeant cADPR antagonist³⁴ capable of inhibiting the release of intracellular Ca^{2+} mediated by CD38/cADPR.³⁵ In order to verify that the concentrations of NAC and 8-Br-cADPR used in our experiments did not affect cell viability, we performed two apoptosis assays. RSV-infected MDDCs were treated with three increasing doses of NAC (0.2, 2 and 10 mM) or 8-Br-cADPR (2, 5 and 20 μ g/ml) and, after 20 hr, we assessed the levels of early apoptosis (annexin V-positive cells) and late apoptosis/dead (annexin V/PI-positive cells) of the cells. Our results have shown that the NAC and 8-Br-cADPR doses utilized did not affect the cell viability (Supporting information, Fig. S1). As shown in Fig. 3, we found that 8-Br-cADPR significantly reduced the expression of all the genes analysed, at 20 hr p.i. The evidence that gene expression inhibition occurred at the later time-point corroborates the notion that CD38 might be induced during the second

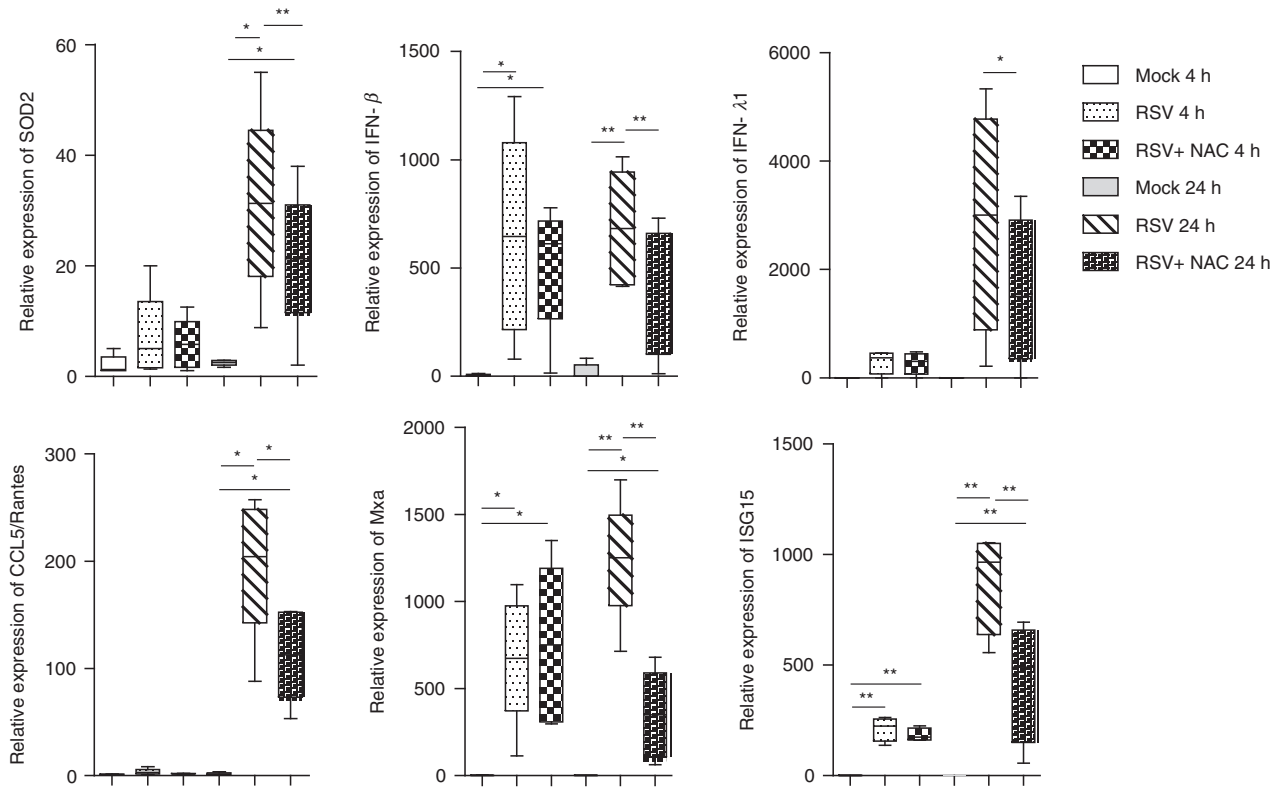


Figure 3. 8-Bromo-cADP ribose (8-Br-cADPR) treatment and genes expression in respiratory syncytial virus (RSV) -infected monocyte-derived dendritic cells (MDDCs). Cells were infected with RSV [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, some samples were pre-treated with 8-Br-cADPR (5 μ g/ml) for 20 min. Mock-infected cells were added as control. After infection, cells were washed, incubated in fresh medium and, where indicated, 8-Br-cADPR was re-added. At the indicated time-points, total RNA was extracted and kinetics of mRNA expression for all genes analysed was evaluated by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR). The mRNA transcripts were normalized with respect to the endogenous reference sample. Data were expressed as fold increase versus mock-treated cells at 4 hr [mean \pm standard error of the mean (SEM) of five experiments]. For all, statistical significance was determined using a two-tailed, unpaired Student's *t* test (**P* < 0.05, ***P* < 0.01).

wave of RSV-induced gene transcription through the action of early produced IFN- β . Overall, these findings identify a putative functional axis linking oxidative stress, IFN/ISGs production and CD38, which might be involved in a positive feedback loop that enhances proinflammatory responses during RSV infection.

Inhibition of CD38 activity by kuromanin decreases RSV-induced responses in MDDCs

To demonstrate that RSV-induced CD38 is catalytically active in MDDCs, we performed a set of experiments to directly measure its enzymatic activity. To this end, we evaluated the accumulation of ADPR, a product of the CD38 catalytic reaction, in the supernatants of RSV-infected cells. The experiments were performed in the presence of the natural anthocyanin kuromanin, a CD38 ADP-ribosylation cyclase inhibitor, which has been shown to inhibit CD38 enzymatic activity.³⁶ After the addition of exogenous nicotinamide adenine dinucleotide (NAD⁺) to RSV-infected MDDCs as substrate

for CD38, we observed an ADPR accumulation. Remarkably, the presence of kuromanin decreased ADPR levels, with about 50% inhibition (Fig. 4a). The inhibitory effect of kuromanin treatment was not caused by decreased CD38 expression (Supporting information, Fig. S2). As previously reported with other flavonoids with similar activity,³⁷ no cell toxicity was observed when MDDCs were exposed to 100 μ M kuromanin for up to 20 hr (not shown). Additionally, to exclude that the inhibitory effect of kuromanin on ADPR accumulation in MDCCs could be related to its interference on RSV entry or replication, the levels of RSV, expressed as number of viral copies/ μ g RNA, in both kuromanin-treated or -untreated RSV-infected cells were analysed. As shown in Fig. 4c, the amount of RSV copies/ μ g RNA had the same order of magnitude, independently of kuromanin addition to the MDCCs cultures.

Given the efficient inhibition on CD38 enzymatic activity exerted by kuromanin, we examined the effects of kuromanin on RSV-induced oxidative and IFN responses.

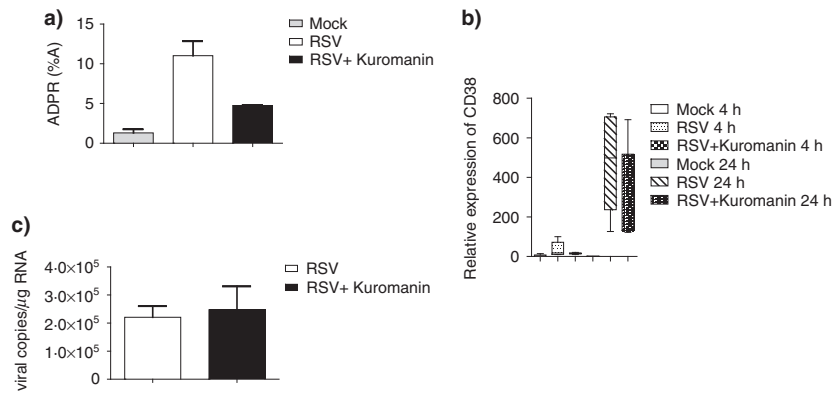


Figure 4. Inhibition of CD38 enzymatic activity by a natural anthocyanin. Cells were infected with respiratory syncytial virus (RSV) [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, some samples were pretreated with kuromanin (100 μ M) for 20 min. Mock-infected cells were added as control. After infection, cells were washed, incubated in fresh medium and, where indicated, kuromanin was re-added. (a) RSV-infected monocyte-derived dendritic cells (MDDCs), at 20 hr, were harvested and incubated with 0.1 mM NGD⁺. Generation of ADPR was measured by high-performance liquid chromatography (HPLC). Quantitative measurements were inferred by comparing the peak area of the samples with the calibration curve for peak area of standard compound. Values were expressed as the percentage of the peak area and are the mean \pm standard error of the mean (SEM) of three independent experiments. (b) RSV-infected MDDCs were harvested at the indicated time-points, and total RNA was extracted. The kinetics of mRNA expression for all genes analysed was evaluated by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mRNA transcripts were normalized with respect to the endogenous reference sample. Data were expressed as fold increase versus mock-treated cells at 4 hr and are mean \pm SEM of five experiments. (c) RSV-infected MDDCs were collected at 20 hr p.i. and viral copies for each sample were determined by RT-PCR (mean \pm SEM of two experiments).

Results are summarized in Fig. 5. Expression of all the genes tested was reduced, and only IFN- λ 1 reduction failed to reach statistical significance. These results confirm that CD38 plays a central role in fostering anti-viral responses.

Discussion

Despite extensive attempts, an RSV vaccine is not yet available.⁶ Analogously, a safe and effective cure for RSV remains elusive: treatment consists of supportive care including supplemental oxygen and mechanical ventilation, while bronchodilators, corticosteroids and ribavirin have failed to show clear benefit.⁷ Immune-prophylaxis with the neutralizing monoclonal antibody palivizumab is used to prevent RSV disease in extremely premature infants or those with congenital heart disease.³⁸ Thus, new intervention strategies are urgently needed.

The current study explored our hypothesis that CD38 is at the centre of a functional axis linking the IFNs response and the oxidative stress induced by RSV. Taking advantage of a well-established preclinical human MDDCs model, we analysed the role of CD38 in the context of RSV infection.

Type I IFNs are classically elicited in response to viral infection; they induce a vast array of ISGs whose activity impairs viral replication in infected cells.³⁹ This cell-intrinsic action plays a crucial role in protecting the lungs from spread of respiratory viruses.⁴⁰ However, type I IFNs also play a key role in the initiation of lung

inflammatory responses by inducing recruitment and activation of immune cells, a process that can cause detrimental immunopathology and contribute to disease severity.⁴⁰ Here, we showed that IFN- β is induced as early as 4 hr p.i. in RSV-infected MDDCs; gene expression analysis also demonstrates that a set of typical ISGs is induced in a second wave of transcription. These genes include MxA and ISG15, both endowed with broad anti-viral activity and markers of RSV infection,^{32,33} and RANTES/CCL5, an inflammatory chemokine associated with increased likelihood of severe asthma after RSV lung disease.⁴¹ In addition, as the emerging role of type III IFNs during in RSV infection,²⁴ the production of IFN- λ 1/IL-29 was also measured. We also found that the CD38 gene is expressed in the second wave of RSV-induced transcription. The association between RSV-induced type I IFNs and CD38 *de-novo* transcription, is witnessed by the finding that blocking type I IFNs significantly reduces RSV-induced CD38 gene expression.

A key aspect of the present study is the finding that the enzymatic activity of CD38 is involved in the anti-viral and proinflammatory responses induced by RSV. cADPR is one of the main products of CD38 catalytic activity and has been recognized as a principal second messenger involved in Ca²⁺ mobilization from intracellular stores.⁴² Moreover, the CD38/cADPR pathway plays an important role in several inflammatory processes.^{43,44} Here, we found that the expression of RSV-induced IFN- β , IFN- λ 1/IL-29, CCL5/RANTES, MxA and ISG15 is reduced significantly in MDDCs by the cADPR analogue 8-Br-cADPR.

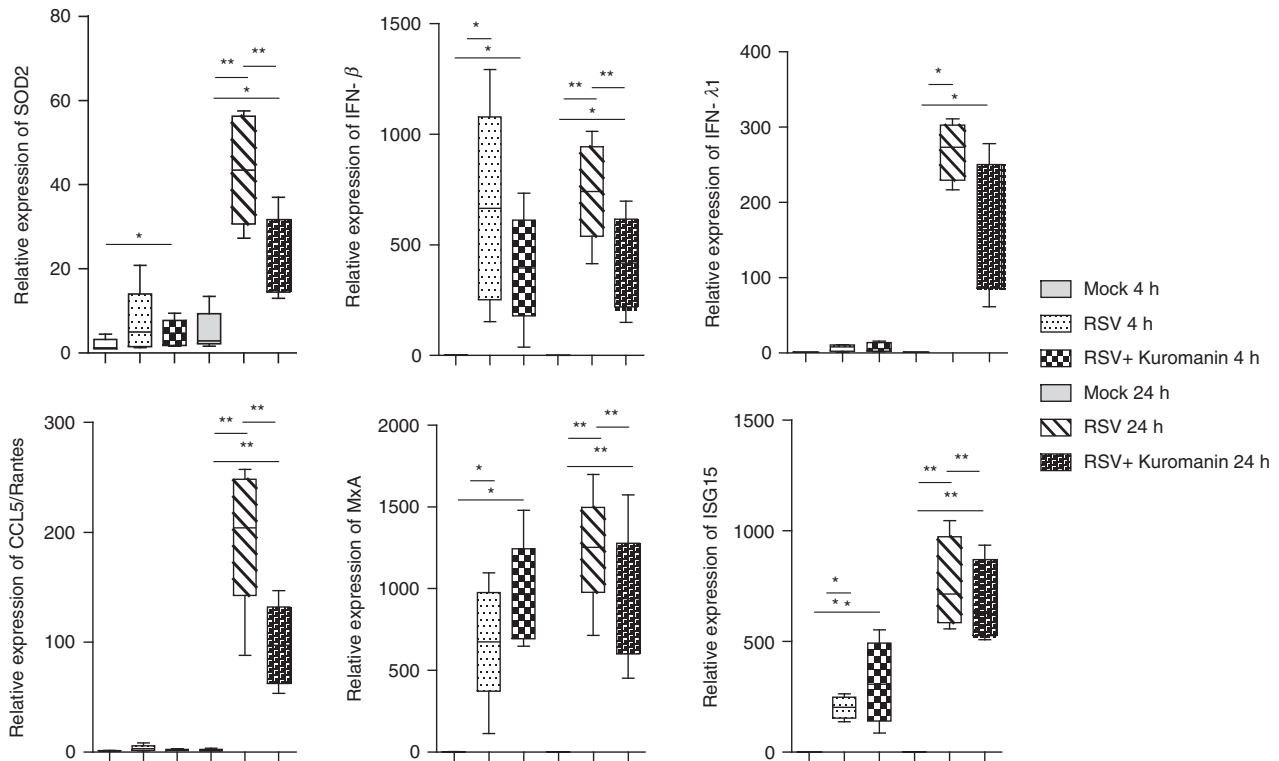


Figure 5. Kuromanin treatment and genes expression in respiratory syncytial virus (RSV)-infected monocyte-derived dendritic cells (MDDCs). Cells were infected with RSV [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, some samples were pretreated with kuromanin (100 μ M) for 20 min. Mock-infected cells were added as control. After infection, cells were washed, incubated in fresh medium and, where indicated, kuromanin was re-added. At the indicated time-points, total RNA was extracted and kinetics of mRNA expression for all genes analysed was evaluated by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR). The mRNA transcripts were normalized with respect to the endogenous reference (human β -actin) sample. Data were expressed as fold increase versus mock-treated cells at 4 hr and are the mean \pm standard error of the mean (SEM) of five experiments. For all, statistical significance was determined using a two-tailed, unpaired Student's *t*-test (**P* < 0.05, ***P* < 0.01).

Based on reports on the key role of oxidative stress in RSV pathology^{16,29} and on the activity of CD38 in other cellular settings, it is tempting to speculate that the IFN/CD38 axis activated by RSV in MDDCs could also be implicated in the regulation of oxidative responses. It is known that CD38 is involved in reactive oxygen species (ROS) production through cADPR.^{27,45} Our results were in agreement with those reported by Hosakote *et al.*;²⁹ indeed, NAC treatment significantly decreased SOD2 gene expression, confirming that increased SOD2 expression is part of an unbalanced oxidative response caused by RSV infection. Notably, when RSV-infected MDDCs were treated with 8-Br-cADPR, SOD2 expression was also markedly and significantly reduced. Moreover, when RSV-infected MDDCs were treated with the anti-oxidant NAC the reduction of RSV-induced IFN- β , as well as ISGs, was verified. Taken together, our results suggest the existence of a functional axis that, when unbalanced during RSV infection, may favour the onset of lung immunopathology; the CD38/cADPR pathway appears at the centre of a functional axis set at the crossroad between type I IFN-dependent anti-viral and proinflammatory responses and

the oxidative burst. We hypothesize that, in RSV-infected MDDCs, CD38-induced cADPR opens ryanodine receptor Ca^{2+} channels, as shown in different settings.⁴⁶ Elevations in Ca^{2+} may induce local production of ROS,⁴⁷ fostering the activation of inflammatory and anti-viral processes (Fig. 6). A directed demonstration that RSV-induced CD38 is enzymatically active derives from the use of NAD^{+} as CD38 substrate. Indeed, after the addition of exogenous NAD^{+} to RSV-infected MDDCs we observed an accumulation of ADPR. Further studies are needed to directly correlate CD38-mediated cADPR with increased levels of intracellular Ca^{2+} and generation of ROS.

A consequence of the present findings is that inhibitors of CD38 enzymatic activities may prove therapeutically useful: an ideal candidate for this purpose is kuromanin.^{48,49} Flavonoids, such as kuromanin, have been shown to inhibit CD38 enzymatic activities.³⁶

Among the available molecules, kuromanin is active in the low micromolar range and has been recently proved to be a potent CD38 inhibitor in chronic lymphocytic leukaemia cells.⁵⁰ At the same time, kuromanin is a natural product and its prospective use for human health

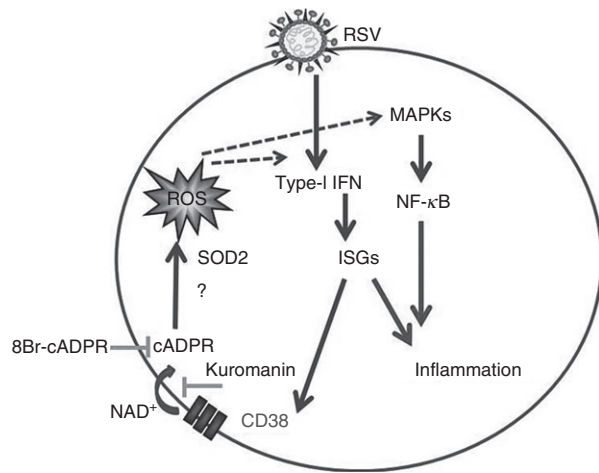


Figure 6. Schematic representation of the proposed functional axis between the CD38/cyclic adenosine diphosphate ribose (cADPR) pathway, type-I interferon (IFN) response and the oxidative burst induced by respiratory syncytial virus (RSV) in monocyte-derived dendritic cells (MDDCs). ROS: reactive oxygen species.

appears to be safe. In RSV-infected MDDCs, kuromanin inhibited CD38 enzymatic activity with approximately 50% reduction, restraining RSV-induced proinflammatory and pro-oxidant responses in MDDCs.

Similarly to what was observed with 8-Br-cADPR, addition of kuromanin decreased the expression of IFN- β , as well as of ISGs and SOD2. In this regard it is important to note that kuromanin is endowed with anti-oxidant activity,⁵¹ thus it is possible to envisage a twofold mechanism employed to prevent the onset of an exaggerated inflammatory response, a specific inhibition of the enzymatic activity of CD38 with consequent reduction in cADPR production; a more general anti-oxidant effect by scavenging free radicals.

Other hints on the mechanisms through which kuromanin may exert its effects come from recent studies. An example comes from a recent study showing that kuromanin inhibits the production of proinflammatory cytokines in intestinal Caco-2 cells through activation of the Nrf-2 pathway.⁵² In this respect, it is known that RSV infection induces a reduction in nuclear and total cellular levels of the NRF2 protein, thus fostering oxidative damage.⁵³ Further studies are required to investigate whether activation of Nrf2 by kuromanin also occurs in RSV-infected MDDCs and whether it could be correlated with inhibition of the CD38/cADPR pathway.

In conclusion, the present study, for the first time, identified CD38 and cADPR as key players in the orchestration of cellular responses to RSV by infected MDDCs and possible targets for interventions aimed at controlling exacerbated lung responses. Kuromanin appears a promising candidate for its intrinsic features.

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Author contributions

I.S. and G.F. designed the study, performed the experiments and wrote the paper; C.S., A.P., P.L. and A.L.H. performed the experiments; F.M. and C.M.A. contributed to study design. The authors thank Simonetta Pietrangeli for excellent technical assistance.

Disclosures

All the authors declare no commercial or financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cells were infected with respiratory syncytial virus (RSV) [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, samples were pretreated with the indicated concentrations of N-acetyl-cysteine (NAC) and 8-Bromo-cADP ribose (8Br) for 20 min.

Figure S2. Cells were infected with respiratory syncytial virus (RSV) [multiplicity of infection (MOI) of 1] for 2 hr. Where indicated, samples were pretreated with Kuromanin (100 μM) for 20 min.