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Respiratory Syncytial Virus Inhibits Granulocyte Apoptosis through a Phosphatidylinositol 3-Kinase and NF-κB-Dependent Mechanism

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Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract disease in children. It is associated with increased neutrophil numbers in the airway. In this study, we assessed whether this ssRNA virus can directly influence granulocyte longevity. By culturing RSV with granulocytes, it was observed that virus delays both constitutive neutrophil and eosinophil apoptosis. Using pharmacological inhibitors, the RSV-induced delay in neutrophil apoptosis was found to be dependent on both PI3K and NF-κB, but not p38 MAPK or MEK1/MEK2 activation. Using blocking Abs and a reporter cell line, we were able to exclude TLR4 as the receptor responsible for mediating RSV-induced delay in neutrophil apoptosis. The antiapoptotic effect was abrogated by preincubation with the lysosomotropic agent chloroquine, indicating the requirement for endolysosomal internalization. Furthermore, addition of ssRNA, a ligand for the intracellular TLR7/TLR8, also inhibited neutrophil apoptosis, suggesting that intracellular TLRs could be involved in induction of the antiapoptotic effect. Using the BioPlex cytokine detection assay (Bio-Rad), we found that IL-6 was present in supernatants from RSV-exposed neutrophils. IL-6 was found to inhibit neutrophil apoptosis, suggesting that there is an autocrine or paracrine antiapoptotic role for IL-6. Finally, RSV treatment of neutrophils resulted in increased expression of the antiapoptotic Bcl-2 protein Mcl-1. Taken together, our findings suggest involvement of multiple intracellular mechanisms responsible for RSV-induced survival of granulocytes and point toward a role for intracellular TLRs in mediating these effects. The Journal of Immunology, 2006, 176: 5529–5537.

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3 Abbreviations used in this paper: RSV, respiratory syncytial virus; LRTD, lower respiratory tract disease; F, fusion; MmLPS, Neisseria meningitidis LPS; MOI, multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide.

The Journal of Immunology
RSVP has been shown to increase cell survival of epithelial cells and monocytes (15–17).

Increased survival of target cells is advantageous for the virus to enable efficient replication before cells are removed by Fas-mediated apoptosis, the natural fate of virally infected cells. In epithelial cells, which are the main target cells for the virus, RSV initiates a productive infection and would benefit from longer cell longevity. In epithelial cells, the prosurvival signal appears to be PI3K and NF-κB dependent (15). However, the mechanism leading to virus-induced activation of these pathways remains unclear.

RSV has been shown to interact with the chemokine receptors CX3CR1 on epithelial cells and TLR4 on monocytes, macrophages, and dendritic cells in mice (2, 18, 19). The RSV G protein may bind the fractalkine receptor for viral entry, and the fusion (F) protein has been found to bind to TLR4 and induce NF-κB activation in epithelial cells. TLR4 is a pattern-recognition receptor belonging to the family of TLRs that can be activated by a variety of pathogens and signal via NF-κB. TLRs activate NF-κB, which, like PI3K, has been shown to influence cell survival in other human cell types, whereas chemokine receptors are not generally involved in providing survival signals (20, 21).

Both neutrophils and RSV are present in the bronchial lumen of children with RSV LRTD. Any mechanism that promotes neutrophil life span may worsen the immunopathologic effects of neutrophils. We have conducted this in vitro study to investigate whether RSV itself contributes to granulocyte survival, and if so, whether this may be the same signaling routes known to be critical for cytokine survival or pattern-recognition receptor-dependent pathways. We have found that incubation of granulocytes with RSV leads to an inhibition of the constitutive apoptotic program. This process was found to be dependent on both PI3K activity and NF-κB activation. Regulation of human neutrophil longevity by a viral pathogen has clinical relevance for understanding disease pathogenesis.

Materials and Methods

Isolation of human neutrophils

Neutrophils and eosinophils were isolated from the peripheral blood of normal healthy volunteers from the blood donor service of the University Medical Center (Utrecht, The Netherlands). Mixed granulocytes were isolated from 10 to 100 ml of blood, which was anticoagulated with 0.32% sodium citrate as described previously (22). In short, blood was diluted 1.4 times with PBS containing 0.32% sodium citrate and 10% human pasteurized plasma protein solution (40 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Paque (Pharmacia Biotech) for 20 min at 2000 rpm. The erythrocytes were lysed in isotonic ice-cold NH4Cl solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 min at 37°C in HEPES-buffered RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 1-glutamine and 0.5% human serum albumin. All preparations contained >97% neutrophils. For eosinophil experiments, eosinophils were separated from neutrophils by negative immunomagnetic selection of the granulocyte fraction after lysis of the erythrocytes, with anti-CD3-, anti-CD14-, and anti-CD16-conjugated microbeads (MACS; Miltenyi Biotech). The purity of eosinophils was >97%.

RSV culture

RSV, an ssRNA virus, was cultured in Hep2 cells cultured in IMDM (Invitrogen Life Technologies) with 1% FCS. After infection of the monolayer with a RSV stock (106 PFU/ml) in a volume ratio of 1:80, culture flasks with a total volume each of 20 ml were placed in a stove at 37°C for 2 days. Syncytia were formed after 36 h, and harvesting was performed after 48 h when large syncytia were present.

Virus-infected medium was pooled in 50-ml tubes and immediately centrifuged at 1200 rpm for 5 min. To further remove all epithelial cell debris, the virus suspension was filtered using a 0.22-μm Stericup filter (Millipore). After that, sterile ice-cold polyethylene glycol 50% (Merck) was slowly added to a final concentration of 10% polyethylene glycol. This mixture was placed at 4°C for 2 h with gently stirring. Thereafter, the ice-cold mixture was centrifugated a 4000 rpm for 30 min to spin down precipitated virus. Pellets were resuspended in IMDM 10% serum (FCS) (Invitrogen Life Technologies), aliquoted, snap-frozen, and stored in liquid nitrogen.

For comparison, RSV stock of 107.78 pfu/ml was purchased from Applied Biosystems, aliquoted in 5 x 104 PFU/100 µl, and stored in liquid nitrogen. These stocks gave identical results.

In some experiments, to exclude effects due to contaminants of the M-100-kDa Millipore filter (Millipore) was used to filter the virus away from the virus prep.

Reagents and Abs

IL-6 and chloroquine were purchased from Sigma-Aldrich. Recombinant human GM-CSF was obtained from Genzyme, and human IL-5 was a gift from GlaxoSmithKline. Purified Neisseria meningitidis LPS (NlPS), isolated from N. meningitidis H44–76 strain, was a gift from Dr. L. Steeghs (23). LY294002 and SB203580 were purchased from Alexons; BAY 11-7082 and Wedelolactone from Calbiochem. Neutralizing azide-free anti-TLR4 (HTA125) Abs were obtained from Riegenn. Polyonalic anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182), and anti-phospho-IκBα (Ser32) Ig were obtained from Cell Signaling Technology. Anti-actin (I-19) and anti-Mcl-1 were purchased from Santa Cruz Biotechnology. The ssRNA40 and PolyU were purchased from Invitrogen Life Technologies. Recombinant human IL-6 was ordered from Chemicon International.

Analysis of apoptosis

For apoptosis assays, neutrophils (4 x 107/ml) were cultured in IMDM 10% FCS for 8–24 h and eosinophils for 48 h in the presence of RSV in multiplicities of infection (MOI) of 1–5. As a control for survival, neutrophils were incubated with GM-CSF (10–10 M) and eosinophils with human IL-5 (10–7 M). When heat-denatured virus was used, the virus was heated for 30 min at 56°C. This heat-inactivated virus failed to form plaques (data not shown).

Apoptosis was determined by Annexin VFITC binding (Bender MedSystems). After staining the cells with Annexin VFITC for 10 min in the dark at room temperature, the cells were washed with annexin binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2) and resuspended in annexin binding buffer containing 1 μg/ml propidium iodide (PI). Cells were analyzed using a flow cytometer (FACSCalibur; BD Biosciences). Viability was defined as the percentage of cells that were double negative for both stains (annexin/PI).

For inhibitor experiments, before the addition of the virus or control stimulus, neutrophils were preincubated for 30 min with the PI3K inhibitor LY294002 (20 μM) and/or the NF-κB inhibitors BAY 11-7082 (0.1 μM) or Wedelolactone (25 μM). To investigate involvement of the endosomal acidification process, cells were preincubated for 30 min with chloroquine (10 μM) a lysosomotropic agent.

Cytokine/chemokine detection

At the end of the 12-h culture period, supernatants were collected and stored at –80°C until analysis. The presence of cytokines was analyzed using the BioPlex system as described by de Jager et al. (24). The following mediators and cytokines were analyzed: IL-6, IL-8, MIP-1α, IL-1β, sICAM, and TNF-α.

Western blot

For analysis of protein presence or protein phosphorylation, neutrophils were stimulated with RSV (MOI 5) for 10, 30, or 60 min. As a control for protein phosphorylation, neutrophils were stimulated with IMLP (1 μM, 2 min) or GM-CSF (10–10 M, 10 min). After stimulation, cells were pelleted by centrifugation and washed twice with ice-cold PBS. For Western blotting with phospho-p38 or phospho-ERK Abs, 1.6 x 106 cells were lysed in 50 μl of boiling sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 2% 2-ME) containing 50 mM DTT and boiled for 5 min at 95°C. For detection of total IκBα or Mcl-1, 4 x 105 cells were lysed in 40 ul of boiling sample buffer with DTT. Protein lysates of equal amounts of cells were separated in 10–15% gradient gels by SDS-PAGE, and Western blotting was performed using the PhastGel system (Amersham Biosciences). After blotting, the polyvinylidene difluoride membranes (Millipore) were incubated in blocking buffer (TBST) supplemented with 5% dried milk. The membranes were incubated overnight with primary Abs in TBST supplemented with 5% BSA. The next day after washing with TBST, they were incubated with secondary Ab, swine anti-rabbit or anti-goat peroxidase-conjugated Ab (DakoCytomation) for 1 h. Hereafter, the...
proteins were visualized with the ECL Plus detection system (Amersham Biosciences).

**NF-kB reporter cell line**

The NF-kB reporter cell line, CHO/CD14/TLR4, coexpressing CD14 and TLR4, was provided by Dr. D. Golenbock (Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA) (25). The cells were cultured in Ham's F-12 medium (Invitrogen Life Technologies), supplemented with 10% defined FBS (Invitrogen Life Technologies), and hygromycin B (Roche), and puromycin (Sigma-Aldrich). The cell line expresses a synthetic gene encoding membrane CD25 fused with the human E-selectin promoter, containing NF-kB binding sites. Activation of NF-kB results in cell surface CD25 expression that can be analyzed by flow cytometry.

**Results**

**RSV delays both neutrophil and eosinophil apoptosis**

Human granulocytes have an intrinsic apoptotic program that can be delayed by survival factors, including hematopoietic cytokines. Although eosinophils can be cultured ex vivo for several days, human neutrophils have a shorter life span even in the presence of survival factors. To determine whether RSV can increase human granulocyte survival, both neutrophils and eosinophils were cultured (0.4 × 10^6 cells/ml) with variable amounts of RSV. The viability of cells was measured by staining with Annexin V-FITC/PI and determining the percentage of cells double negative for both stainings by FACS. Cells that were single positive for annexin were regarded as early apoptotic, and double-positive cells were regarded as late apoptotic. Cells that were single positive for PI were considered to be necrotic and were <1% in all experiments. Neutrophil apoptosis was substantially delayed at 8, 12, 18, and 24 h (Fig. 1A), whereas eosinophil apoptosis was delayed at both 24 and 48 h (Fig. 1B). The viability of cells increased with higher concentrations of RSV for both cell types. To determine whether the intact virus was critical, filtered virus was used (see Materials and Methods). The virus-free eluate did not have the antiapoptotic effect when incubated with either neutrophils or eosinophils, demonstrating that the antiapoptotic effect was virus mediated (Fig. 2A and B). These data clearly demonstrate that RSV was capable of inhibiting the intrinsic apoptotic program of human granulocytes. The effect was observed with relatively low concentrations of virus. This might have been mediated by 1) a productive infection that would rapidly increase the amount of virus particles, 2) an extremely sensitive virus-receptor interaction, or 3) induction of the antiapoptotic effect by viral component as well. The latter can be investigated by heat inactivation of the viral preparation. Incubating granulocytes with heat-denatured RSV (MOI 5 before heat inactivation at 56°C) increased viability of neutrophils at 12 h, compared with controls, to the same extent as viable RSV MOI 5 (Fig. 2C). Taken together, these results demonstrate that RSV can inhibit granulocyte apoptosis, and that this effect does not require the presence of live virus.
PI3K is required for RSV-induced inhibition of neutrophil apoptosis

Although a variety of intracellular signaling pathways has been reported to be activated by survival factors such as GM-CSF, activation of PI3K has been shown to be critical for neutrophil survival. To determine whether PI3K activation also is necessary for RSV-induced inhibition of granulocyte apoptosis, neutrophils were preincubated for 30 min with the specific PI3K-inhibitor, LY294002. Addition of LY294002 decreased the survival of neutrophils incubated with RSV (Fig. 3A). Inhibition of PI3K completely inhibited GM-CSF-induced survival, in accordance with previously published studies (data not shown and Refs. 9–13).

MAPKs also have been reported to play a role in survival of granulocytes in response to antiapoptotic stimuli other than cytokines (26). However, cell viability was unaffected when cells were cultured in the presence of RSV and MAPK inhibitors U0126 (MEK1/MEK2 inhibitor) and SB203580 (p38 inhibitor), compared with RSV alone (Fig. 3A). Interestingly, a small increase in p38 phosphorylation was observed after incubating neutrophils with RSV for 60 min, which was not observed for ERK1/ERK2 (Fig. 3, B and C).

RSV-induced survival requires internalization and activates NF-κB independently of TLR4

An interaction has been described between RSV and TLR4 (18, 27). TLR4 has been shown to be present on human neutrophils (28), and its activation has been demonstrated to increase neutrophil life span (29). To determine whether TLR4 could be involved in RSV signaling leading to a delay in neutrophil apoptosis, neutrophils were preincubated for 30 min with HTA 125, a neutralizing Ab against human TLR4. Blocking TLR4 had no effect on RSV-induced survival (Fig. 4A).

To further confirm that RSV does not directly activate TLR4, we used an NF-κB reporter cell line, CHO/CD14/TLR4, in which TLR4 activation induces cell surface CD25 expression. TLR4 reporter cells were stimulated with RSV and NmLPS was used as a control. Whereas NmLPS induced CD25 expression, RSV did not (Fig. 4B). In conclusion, we found no evidence that RSV-induced cell survival occurs via interaction with TLR4.

RSV is a ssRNA virus, and recently, TLR7 and TLR8 have been identified as receptors for ssRNA (30). These intracellular TLRs require endosomal internalization for activation. To investigate whether endolysosomal internalization of RSV is required to establish the antiapoptotic effect, lysosomotropic agents, such as

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** RSV-induced neutrophil survival is PI3K dependent. A, Neutrophils (0.4 × 10^6/ml) were preincubated for 30 min with pharmacological inhibitors of PI3K (20 μM LY294002), p38 MAPK (10 μM SB203580), and MEK (10 μM U0126) as indicated. Cells were then incubated with RSV (MOI 5) for 12 h and apoptosis measured as described in Materials and Methods. Data represent five independent experiments ± SEM. The effect of the inhibitors on RSV-induced increase in viability was analyzed using one-way ANOVA corrected with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *p < 0.05; **p < 0.01; ***p < 0.001. B and C, Neutrophils (0.4 × 10^6/ml) were stimulated with FMLP (10^{-6}M; 2 min), GM-CSF (10^{-10}M; 10 min), or RSV (MOI 5) as indicated. Cell lysates were prepared and analyzed by Western blotting for ERK1/ERK2 or p38 MAPK activation as detailed in Materials and Methods. Blots were reprobed with anti-actin Ab. Data are representative of four independent experiments.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** RSV-induced delay in neutrophils apoptosis is not dependent on TLR4 activation. A, Neutrophils (0.4 × 10^6/ml) were pretreated with TLR4 blocking Ab (HTA125; 10 μg/ml) before incubation with GM-CSF (10^{-8}M) or RSV for 12 h as indicated. Viability was measured as described in Materials and Methods. The effect of HTA125 was analyzed using one-way ANOVA corrected with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *p < 0.05; **p < 0.01; ***p < 0.001. B, A readout system for TLR4 activation, a CHO cell line overexpressing TLR4 with CD25 expression under NF-κB control, was used. Cells were incubated with RSV (MOI 5) or NmLPS for 18 h and cells analyzed for CD25 expression as detailed in Materials and Methods. Data represent three independent experiments ± SEM. Data in this graph were analyzed using ANOVA statistics with Dunnett’s posttest for multiple comparisons (all points vs control) (*p < 0.05).
chloroquine, can be added to prevent endosomal acidification (30). This approach has been previously used to identify ssRNA as the responsible component for increasing IFN production by influenza virus in human dendritic cells (30). Preincubation of granulocytes with chloroquine completely abrogated the antiapoptotic effect of the RSV, while having no inhibitory effect on GM-CSF-stimulated survival (Fig. 5A). This demonstrates that endosomal acidification is required for RSV-induced survival. It suggests that, similar to influenza virus, RSV also requires endosomal internalization for the observed effect and intracellular TLRs may be involved. TLR3, TLR7, and TLR8 have been linked with viral infection (31, 30). TLR7 and TLR8 are quite similar, and of the two, TLR8 may be most relevant for human sensing of viral ssRNA (32). Because we have demonstrated that replication is not required for the RSV-induced antiapoptotic effect, it was investigated whether TLR7 and TLR8 ligands may have a similar effect on granulocyte viability. Indeed, ssRNA40 and polyU, known ligands for TLR7 and TLR8, inhibited neutrophil apoptosis, although not to the same extent as RSV itself (Fig. 5B).

If TLRs are involved in induction of the antiapoptotic effect of RSV, NF-κB activation should be observed. In a variety of cell types, genes critically regulating cell survival are under transcriptional control of NF-κB, a ubiquitously expressed heterodimeric transcription factor (33, 34). Activation of NF-κB depends on degradation of I-κB, which normally sequesters NF-κB in the cytoplasm, inhibiting its function. To determine whether NF-κB was activated by RSV exposure, we analyzed I-κBα expression by Western blotting. Indeed, expression of I-κBα was found to be decreased after 10 and 30 min of RSV incubation, compared with the control situation (Fig. 6A). Furthermore, inhibition of NF-κB by two distinct pharmacological inhibitors resulted in a significant decrease in the RSV-enhanced cell viability (Fig. 6B). BAY 11-7082 irreversibly and selectively inhibits the TNF-α-inducible phosphorylation of I-κBα without affecting constitutive autophosphorylation (35, 36). Wedelolactone inhibits NF-κB-mediated gene transcription in cells by blocking the phosphorylation and degradation of IκBα (37).

This demonstrates that interaction of neutrophils with RSV leads to I-κBα degradation resulting in NF-κB activation. Taken together, RSV induces NF-κB activation, which is at least partially responsible for the antiapoptotic viral effect.

**FIGURE 6.** RSV-induced neutrophil survival is NF-κB dependent. A, Neutrophils were incubated with RSV (MOI 5) for the times indicated, and cell lysates prepared and analyzed for I-κB expression as described in Materials and Methods. Blots were reprobed with anti-actin Ab. Data are representative of four independent experiments. B, Neutrophils (0.4 × 10⁶/ml) were preincubated for 30 min with NF-κB inhibitors BAY 11–7082 (0.1 μM) or wedelolactone (25 μM), in the absence or presence of LY294002 (20 μM) as indicated. Subsequently, cells were treated with RSV (MOI 5) for 12 h and viability measured as described in Materials and Methods. Data represent four independent experiments ± SEM. The effect of the inhibitors was analyzed using one-way ANOVA corrected with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**RSV-induced IL-6 production increases neutrophil viability**

The possibility exists that RSV delays neutrophil apoptosis through cytokine production regulated by PI3K- and NF-κB-dependent pathways. To test this hypothesis, we analyzed the presence of cytokines in the supernatants of granulocytes after 12 h of RSV incubation by BioPlex (see Materials and Methods). Only IL-6 was found to be present in significantly higher amounts in RSV-treated cells, compared with controls and GM-CSF stimulated cells (Fig. 7A). IL-8, MIP-1α, IL-1β, sICAM, and TNF-α also were analyzed but not found to be significantly increased after 12 h of incubation with RSV (data not shown). Interestingly, IL-6

**FIGURE 5.** Endolysosomal internalization is required for the RSV-induced increase in neutrophil viability. A, Neutrophils were pretreated with or without the lysosomal agent chloroquine (10–10 M) for 30 min before addition of GM-CSF (10–10 M) or RSV (MOI 5). Viability was measured after 12 h as described in Materials and Methods. Data represent four independent experiments ± SEM. The effect of the inhibitors was analyzed using one-way ANOVA corrected with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, Neutrophils were treated with GM-CSF (10–10 M), RSV (MOI 5), or increasing concentrations of poly-U or RNA-40 as indicated. Viability was measured after 12 h as described in Materials and Methods. Data represent three independent experiments ± SEM. Data in this graph were analyzed using ANOVA statistics with Dunnett’s posttest for multiple comparisons (all points vs control) (⁎, p < 0.05).
has been observed previously to be up-regulated in diverse RSV infection models, although the cellular source of cytokine production was not defined (38, 39). IL-6 has been shown to be transcriptionally up-regulated in an NF-κB-dependent manner (40, 41). To determine whether RSV-induced IL-6 production also is dependent on NF-κB, we determined the effect of NF-κB inhibitors on IL-6 levels in neutrophils incubated with RSV (Fig. 7A). We observed decreased levels of IL-6 in supernatants of cells preincubated with NF-κB inhibitors before the addition of RSV. The antiapoptotic effect of IL-6 in neutrophils has been shown by others but was confirmed by us at 12 h of incubation (Fig. 7A). As we have shown in Fig. 3A, the antiapoptotic effect of RSV is PI3K dependent. We tested whether the antiapoptotic effect of IL-6 on granulocytes is similarly regulated. Inhibition of granulocyte apoptosis by IL-6 was abrogated by preincubation with LY294002, indicating PI3K dependence (Fig. 7C). To determine whether IL-6 also could influence on NF-κB activation, we analyzed IκB expression. After incubation with IL-6 for 30 min, IκB degradation was observed (Fig. 7D).

Taken together, these data demonstrate that RSV can induce IL-6 production in human neutrophils, and that this cytokine is able to repress the intrinsic apoptotic pathway.

RSV induces increased expression of the antiapoptotic protein, Mcl-1

Pro- and antisurvival signals in cells often are translated into differences in the balance between proteins of the pro- and antiapoptotic Bcl-2 family (42). Antiapoptotic Bcl-2 family members are known to be relatively unstable in granulocytes, being subject to proteasomal degradation (43). GM-CSF has been shown previously to increase the expression of Mcl-1, a proapoptotic Bcl-2 family member (43, 44). To analyze the effect of RSV on Mcl-1 protein expression, we incubated cells with RSV for 2 h and determined Mcl-1 expression levels. We found increased expression of Mcl-1 after 2 h of incubation with RSV, compared with control samples (Fig. 8A). This suggests that RSV is indeed able to reg-

FIGURE 7. RSV induces neutrophil IL-6 production in an NF-κB-dependent manner. A, Neutrophils (0.4 × 10⁶/ml) were treated with RSV (MOI 5) in the absence or presence of BAY 11-7082 (0.1 μM) or wedefolactone (25 μM) for 12 h. IL-6 production was measured by BioPlex cytokine detection assay as described in Materials and Methods. The effect of the inhibitors was analyzed using one-way ANOVA corrected for with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, Neutrophils were treated with increasing concentrations of IL-6 as indicated and apoptosis measured after 12 h. Data are representative of five independent experiments. Data in this graph were analyzed using ANOVA statistics with Dunnett’s posttest for multiple comparisons (all points vs control) (*, p < 0.05). C, Neutrophils were incubated with IL-6 (500 ng/ml) or GM-CSF (10⁻¹⁰M) with and without preincubation with LY294002 (20 μM). Apoptosis was measured after 12 h. Data are representative of three independent experiments. The effect of LY294002 on IL-6- and GM-CSF-induced cell viability was analyzed using one-way ANOVA corrected with Bonferroni’s posttest for multiple comparisons. Levels of significance are shown as *, p < 0.05; **, p < 0.01; ***, p < 0.001. D, Neutrophils were incubated with IL-6 (500 ng/ml) for the times indicated and cell lysates prepared and analyzed for IκB expression as described in Materials and Methods. Blots were reprobed with anti-actin Ab.

FIGURE 8. Regulation of Mcl-1 expression in neutrophils by RSV and IL-6. A, Neutrophils were left untreated, or incubated with RSV (MOI 5) or GM-CSF (10⁻¹⁰M) for 2 h. Cell lysates were prepared and analyzed for Mcl-1 protein expression as described in Materials and Methods. Blots were reprobed with anti-actin Ab. Data are representative of three independent experiments. B, Neutrophils were preincubated (30 min) with and without LY294002 (20 μM) and then stimulated with IL-6 (500 ng/ml) or GM-CSF (10⁻¹⁰M) for 4 h. Cell lysates were prepared and analyzed for Mcl-1 protein expression. Blots were reprobed with anti-actin Ab.

FIGURE 9. Inhibition of neutrophil apoptosis by RSV. RSV activates PI3K, NF-κB, and p38 through internalization and interaction with intracellular TLRs. This leads to PI3K-mediated Mcl-1 stabilization resulting in inhibition of neutrophil apoptosis. Furthermore, RSV-mediated activation of NF-κB results in IL-6 production that may have autocrine and/or paracrine effects, further prolonging neutrophil life span.
ulate expression of antiapoptotic protein Mcl-1, which also is likely to be involved in delaying granulocyte apoptosis (Fig. 9). We also investigated whether IL-6, potentially acting as an RSV-induced survival factor, could similarly regulate Mcl-1 levels in neutrophils. We observed that IL-6 indeed induces Mcl-1 in granulocytes within 3 h of incubation (Fig. 8B) in a PI3K-dependent manner.

Discussion
In the current study, we have found that incubation of granulocytes with RSV leads to a delay in the constitutive apoptotic program. The molecular mechanism is dependent on both PI3K and NF-κB activation. IL-6, which was present in RSV-treated granulocyte supernatants, has been shown to induce apoptotic delay in human neutrophils and inhibition of NF-κB caused inhibition of IL-6 production in these cells. Therefore, it is tempting to speculate on an autocrine or paracrine role for IL-6 in virus-induced survival of human granulocytes. IL-6 production by RSV also has been confirmed by others. Arnold et al. (45) found increased IL-6 levels in supernatants from neutrophils incubated with RSV. In those experiments, IL-6 levels were found to be maximal at 4 h and absent at 24 h. Furthermore, recent studies have demonstrated increased IL-6 levels in BAL from both RSV-infected mice as well as infants with RSV-induced bronchiolitis (38, 39). This supports a potential role for production of IL-6 by granulocytes in RSV-induced disease pathogenesis. Rhinovirus has also been shown to induce IL-6 production through an NF-κB-dependent mechanism in lung stromal cells (34). The delay in constitutive apoptosis by RSV also is reflected by increased expression of the antiapoptotic protein Mcl-1 (Fig. 8A). Mcl-1 mRNA and protein have a very short half life (43, 44). Because proapoptotic proteins are much more stable, they tend to prevail in the absence of survival signals that increase antiapoptotic protein and mRNA stability or induce de novo synthesis (46). IL-6 also was found to regulate Mcl-1 expression (Fig. 8B), and thus RSV-induced IL-6 production could be responsible for extending the duration of Mcl-1 expression after exposure to RSV. Others have shown that GM-CSF induced survival and enhanced Mcl-1 stability by inhibition of proteasomal degradation. This required both activation of the PI3K and p44/42 ERK/MEK pathway (43). In our experiments, RSV-induced survival also was decreased by incubation with the PI3K inhibitor LY294002. Analysis of p38 MAP kinase also revealed an increased phosphorylation of p38 after RSV exposure (Fig. 3C). However, this is unlikely to contribute to granulocyte survival because SB203580 treatment did not effect neutrophil apoptosis (Fig. 3A). Nonetheless, RSV-induced p38 phosphorylation suggests that other granulocyte functions may be modulated by the virus, such as NADPH oxidase activation (47, 48). Furthermore, p38 activation, similar to NF-κB, is a hallmark of TLR activation, indirectly providing further support for a role for these receptors in RSV-mediated intracellular signaling.

This is the first study to address the direct influence of RSV on granulocyte survival. The actual degree of interaction that takes place between virus and cells of the immune system is hard to determine. However, the fact that our findings demonstrate that heat-inactivated virus also is able to delay neutrophil apoptosis may have clinical implications because virus-infected debris is known to be present in the lumen of the airways. RSV LRTD occurs mainly in children <1 year of age; the median age of children with RSV LRTD is 3 mo (49). In this study, we have used neutrophils from normal healthy adult donors. However, evidence is accumulating that neonatal neutrophil survival may be prolonged, partly due to a reduced responsiveness to Fas ligand (50). It has been argued that neonatal granulocytes with delayed apoptosis may better retain their functionality over time in response to external stimuli than adult neutrophils (51). Thus, agents such as RSV interfering with constitutive apoptosis may even have a more pronounced effect on neonatal neutrophils.

Pattern recognition receptors are evolutionary designed to recognize microbial agents of both bacterial and viral origin and several TLRs that have recently been linked to activation of immune cells are expressed on granulocytes. Microbial agents present at inflammatory sites may affect survival by interaction with TLRs, which are known to activate NF-κB. Nagase et al. (52) have shown a variety of TLRs to be expressed on mRNA level in both neutrophils and eosinophils. By stimulating eosinophils with the available ligands for TLRs, only TLR7 and TLR8 ligands induced eosinophil activation. RSV is an enveloped ssRNA virus. Its G or F protein is thought to bind to a membrane receptor, resulting in entry and possibly induction of activation of intracellular signaling pathways. An interaction has been described between RSV and TLR4 (18, 27). Furthermore, TLR4 has been shown to be present on human neutrophils (28), and its activation has been demonstrated to increase neutrophil life span (29). However, Ehl et al. (53) found no critical role for TLR4 in the resistance of mice to RSV infection. RSV F protein has been shown to interact with TLR4 on epithelial cells, and the expression of TLR4 is up-regulated by RSV infection. In vivo, monocyte TLR4 expression is up-regulated during RSV infection in children (54). Furthermore, it has been shown that macrophages from TLR4-deficient and TLR4-mutant mice strains demonstrated no production of IL-6 in response to incubation with RSV F protein in contrast with wild-type mice (18). Although this receptor also is expressed on neutrophils, we have used neutralizing Abs against TLR4 to study whether TLR4 was involved in delaying granulocyte apoptosis. TLR4 Abs did not interfere with the effect elicited by RSV incubation. However, there remains some controversy as to the relevance of TLR4 in RSV-induced disease. The differences in early RSV replication seen between TLR4-deficient and wild-type mice were shown to be attributable to an additional defect in the IL-12R that these mice also have and not to the absence of TLR4 (53). Furthermore, in experiments with Sendai virus, which has a very similar F protein as RSV, no evidence of interaction between TLR4 and virus could be established (55). In our study, we found no evidence for TLR4 in enhancement of neutrophil life span by RSV. Furthermore, we have performed experiments, using a TLR4 reporter-transfected CHO cell line (25). This cell line allows screening for TLR4 ligands; however, our data demonstrate that RSV is unable to activate TLR4 on these cells.

Currently, options to test the hypothesis that intracellular TLRs could be involved are limited. It was published recently that stimulation of neutrophils with certain synthetic TLR ligands induces a delay in apoptosis that requires PI3K and NF-κB activation leading to increased levels of antiapoptotic Bcl-2 family members (56). It was shown that LPS (a ligand for TLR4) and R-848 (a ligand for TLR7 and TLR8) can induce a delay in apoptosis of neutrophils. In addition to this, we now show that RSV, a biologically relevant ligand, also can enhance neutrophil life span most likely through activation of TLRs. Interaction of all TLRs with their respective ligands leads to activation of the transcription factor, NF-κB. However, we have shown inhibition of the RSV antiapoptotic effect by incubation with chloroquine, suggesting the necessity of endosomal internalisation to induce the effect. Unfortunately, no specific inhibitors are available to distinguish between intracellular TLRs in primary human cells. Diebold et al. (30) also found the involvement of endosomal ssRNA signaling in inducing IFN production from plasmacytoid dendritic cells. Our data suggest that indeed different ssRNA viruses may trigger intracellular TLRs and
induce an innate immune response. Whether or not an increased life span is to the benefit of the host is unclear. It also may be an aspecific immune evasion technique evolved by viruses that may even be harmful to the host when occurring in neutrophils by increasing pathology to the airway.

This is the first study to determine the direct effect of RSV on human granulocyte survival and signaling. Our findings suggest the involvement of multiple intracellular mechanisms responsible for RSV-induced survival of granulocytes with a role for IL-6-production in response to viral challenge. RSV is a complex ligand, and different components of the virus (e.g. F protein, G protein, and ssRNA) may be involved in activation of survival pathways. The potential involvement of intracellular TLRs suggests that our findings will be relevant for other respiratory viruses as well. Neutrophils play a role in the immunopathology during RSV disease and are continuously recruited from the bone marrow and the peripheral blood to the lungs. Extension of neutrophil life span both by viable and nonviable RSV particles therefore potentially contributes to the persistence of functionally active neutrophils in the lungs of infected infants. Understanding the molecular mechanisms underlying the prosurvival effects of RSV may lead to development of novel strategies for treating viral LRTD.

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Disclosures
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References


