Neutrophils in respiratory syncytial virus infection: more trouble than they're worth?

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Abstract

Lower respiratory tract infections by respiratory syncytial virus (RSV) impose a large burden of disease on infants, the immunocompromised, and the elderly. In RSV infection-induced bronchiolitis, the lungs are flooded with neutrophils. Whether neutrophils contribute to the control of viral replication in RSV infection, or only exacerbate immunopathology, remains an open question. Traditionally, neutrophils were only thought to contribute to the elimination of extracellular bacteria and fungi. However, the classical view of neutrophils as unsophisticated microbe killers is obsolete. Neutrophils are increasingly recognised as important contributors to innate and adaptive immunity with a plethora of regulatory functions. Neutrophils stimulate, polarise, as well as suppress T cell responses, promote antibody production, support NK cell homeostasis, and contribute to the resolution of inflammation. Neutrophil extracellular traps (NETs) are imbued not only with antibacterial and antifungal, but also with antiviral activity. Even the classical neutrophil effector mechanisms, i.e. phagocytosis and degranulation, can be brought to bear against viral infection. Moreover, neutrophils are well equipped to detect viruses. Thus, striking new roles for neutrophils in immunity have emerged. This review examines the recent literature on the possible contributions of neutrophils to antiviral defence, in particular against RSV.

Introduction

Respiratory syncytial virus (RSV) is a ubiquitous, seasonal human pathogen that, upon infection of the upper respiratory tract, causes cold-like symptoms in most healthy adults and children. Lower respiratory tract infections (LRTI) by RSV, however, are a major cause of morbidity and mortality among infants, immunocompromised adults, and the elderly. Of all infants younger than 1 year, 1-2% are admitted into hospital suffering from RSV infection-induced bronchiolitis, making it the foremost cause of infant hospitalisation[1,2]. Rates of RSV-induced pneumonia in bone marrow transplant patients prior to engraftment may be as high as 80%, with pneumonia-associated mortality of 70-80%[3]. On average, RSV will infect 5-10% of nursing home residents, causing pneumonia in 10-20% and death in 2-5% of infected per year[3].

RSV is an enveloped virus of the *Pneumovirus* genus in the *Paramyxoviridae* family with a non-segmented, negative-, single-stranded RNA genome of 15,2 kb that contains 10 genes which encode 11 proteins. The principal target cells of RSV infection are the type I and II alveolar pneumocytes and bronchiole surface epithelial cells, and possibly alveolar macrophages[4]. In addition to direct damage to the airways from viral replication, lung pathology is exacerbated by host immunity[5]. Neutrophils in particular appear to contribute to the immunopathology in severe RSV bronchiolitis, where they account for approximately 80% of infiltrating cells measured in bronchoalveolar lavage (BAL) fluid[6,7]. Due to poor specificity of neutrophil effector mechanisms, e.g. release of proteases, neutrophil activation invariably induces bystander damage to surrounding tissue[8].

Neutrophils, the most abundant leukocytes in human circulation, are classically portrayed as unsophisticated, first-line, suicidal foot soldiers whose role is limited to the engulfment and subsequent elimination, at any cost, of invading extracellular microbes. A surge in discoveries over the last two decades has rendered this view invalid[9-11]. Crosstalk of neutrophils with other immune cells, via cytokine production and direct cell-cell interactions, on-site as well as following migration to lymphoid tissues, highlights

the previously unrecognised complexity and regulatory role of neutrophils[10,11].

Hence, there is a budding realisation that neutrophils may aid in the defence against intracellular pathogens, including viruses. While the neutrophil infiltration observed in severe RSV bronchiolitis is excessive, neutrophil recruitment may nonetheless be a functional response in RSV infection. In this literature review, recent advances in our understanding of neutrophil-mediated antiviral defence against RSV will be discussed. For further background information on neutrophils, the reader is referred to a number of excellent recent reviews[9-12].

Literature review strategy

The MEDLINE database was queried for articles published from 2010 to 2014 that contained the terms "neutrophil" and "virus" (or the PubMed thesaurus-defined equivalents). Publications in the thus-generated list that were deemed of the greatest relevance and interest to this review were selected. In addition, articles referenced in the selected publications were searched for further relevant literature.

Detection of RSV by neutrophils

Neutrophils express a wide variety of pattern-recognition receptors (PRRs) that detect pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), including the surface- and endosome-expressed Toll-like receptors (TLRs) and cytosolic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs)[13-15]. Except for TLR3, neutrophils express all TLRs. Despite the importance of neutrophils to the immunopathology of RSV bronchiolitis, little has been reported on the direct recognition of RSV-infected cells and virions by neutrophils. TLR2, TLR4, TLR6, TLR7/TLR8, RIG-I, MDA5, and NOD2, all of which are expressed by neutrophils, are implicated in the recognition of RSV. It is therefore feasible that neutrophils may detect RSV via these PRRs. Nonetheless, direct experimental verification still beckons.

TLR4/CD14/MD-2 complex

TLR4 and coreceptor CD14 were found to mediate the recognition of purified RSV fusion (F), but not attachment (G) or nucleocapsid (N), protein by monocytes[16]. A subsequent study utilising HEK293T transfectants demonstrated that RSV F protein recognition additionally depends on MD-2 expression[17]. By extension, neutrophils, which express TRL4, CD14, and MD-2, are likely to recognise F protein on the surface of RSV-infected cells and virions. However, experimental verification of TLR4/CD14/MD-2 complex-mediated recognition of RSV by neutrophils is required.

TLR4 expression on neutrophils obtained from blood and BAL samples of infants with severe RSV bronchiolitis is reduced, compared to ventilated, non-infected controls, despite the higher state of activation of RSV patient-derived neutrophils, as indicated by comparatively increased CD11b expression[18]2. Upon cytokine- or PAMP-induced activation, neutrophils normally increase TLR4 expression[19]. A defect in TLR4-mediated recognition of RSV by neutrophils may represent a predisposing factor for severe RSV bronchiolitis[18].

By examining the possible correlation between the severity of disease and TLR4 protein levels in blood neutrophils (for ethical reasons, BAL samples can not be obtained in mild cases), further support for a clinical role thereof may be obtained. Moreover, two TLR4 gene polymorphisms are reportedly associated with severe RSV bronchiolitis, as is impaired TLR4 signalling by peripheral blood mononuclear cells (PBMCs) in response to the prototypical agonist, lipopolysaccharide (LPS)[20,21]. However, the association of TLR4 gene polymorphisms with severe RSV bronchiolitis was not confirmed in other studies[22,23]. Nonetheless, there may be a role for TLR4-mediated recognition of RSV by neutrophils in disease development that merits further investigation.

TLR2/6 heterodimer

In a screening study, the response of TLR1-, TLR2-, TLR4-, and TLR6-deficient mice to infection with RSV was examined[24]. Each of

these TLRs is expressed by neutrophils[13]. Of note, TLR2 forms heterodimers with TLR1 and TLR6. Macrophages obtained from TLR2- and TLR6-deficient mice, but not TLR1-deficient mice, release less inflammatory mediators in response to live and UV-irradiated RSV virions. Further, these mice, as well as TLR4-deficient mice, show impaired control of RSV infection, as indicated by increased viral loads in the lungs at 4 days post-infection. In addition, TLR2-deficient mice show reduced dendritic cell (DC) activation and neutrophil migration to the lungs. Therefore, the TLR2/6, but not the TLR1/2, heterodimer is involved in the recognition of RSV infection; the elicited immune response that controlled viral replication included the recruitment of neutrophils[24]. However, the moiety detected by TLR2/6 in this setting remained undetermined.

TLR7/TLR8

Exposure to RSV inhibits neutrophil apoptosis; the detection of RSV by TLR7 and TLR8 is implicated in this effect[25]. TLR7 (mouse neutrophils) and TLR8 (human neutrophils) recognise single-stranded RNA present in endosomes[13]. Endolysosomal internalisation and acidification are required for the inhibition of apoptosis, but active RSV replication is not; a blocking antibody was used to rule out involvement of TLR4 signalling. Treatment with known TLR7 and TLR8 agonists induces a similar, though reduced, anti-apoptotic effect compared to RSV. This suggests that human neutrophils detect phagocytosed RSV RNA via TLR8[25]. However, others suggest that the anti-apoptotic effect depends on a soluble factor produced by contaminating CD14+ monocytes in the neutrophil preparations[26]. TLR7-deficiency exacerbates RSV-induced lung injury in mice. This appears to depend on increased IL-23 and decreased IL-12 production by DCs, thereby skewing the adaptive immune response from a protective T helper 1 (Th1) to a pathogenic Th17 response[27]. Thus, while TLR7/TLR8 appears capable of detecting RSV, the role of TLR7/TLR8 on neutrophils for RSV recognition remains unclear.

RIG-I & MDA5

IFN-β promoter stimulator (IPS-1) acts as adaptor for RIG-I and melanoma differentiation–associated gene 5 (MDA5). Both are RLRs that recognise cytosolic double-stranded RNA and stimulate the production of antiviral type I interferons (IFNs). During RSV infection, IPS-1-deficient mice show an abrogated local and systemic IFN-β response, reduced viral clearance, and increased inflammation that includes enhanced neutrophil recruitment[28]. This casts some doubt on whether RIG-I or MDA5-mediated recognition of RSV is import to neutrophils.

Bone marrow chimeras, with IPS-1-deficiency in either the haematopoietic or non-haematopoietic compartment, demonstrate that although the effect is strongest with a general IPS-1 deletion, expression in either the immune or non-immune cell compartment partially rescued viral clearance[28]. Therefore, recognition of RSV by RIG-I and MDA5 is, at the very least, not restricted to neutrophils. Lung epithelial cell-expressed RIG-I and MDA5 appear largely responsible for the IPS-1-dependent antiviral effect.

Recently, RSV was found to infect and undergo transcription in neutrophils[29]. Since RSV is a negative-stranded RNA virus, the generation of RSV messenger (m)RNA requires the formation of double-stranded RNA intermediates that may be detected by RIG-I or MDA5. Neutrophils express significant levels of cytosolic RIG-I and MDA5. In response to transfection with poly(I:C), a RIG-I and MDA5 agonist, human neutrophils activate an antiviral transcription programme that includes type I IFNs[30]. (TLR3, another poly(I:C)-sensing receptor, is not expressed by neutrophils). Therefore, the RIG-I and MDA5 in RSV-infected human neutrophils may recognise the double-stranded RNA intermediates of RSV and induce the production of antiviral type I IFNs.

The capability of RSV to infect neutrophils could be important to the role that neutrophils play during infection, compared to viruses that lack this capacity. As noted above, it provides an additional means for neutrophils to detect the virus, as well as a way for the virus to directly

modulate neutrophil functionality. For instance, RSV nonstructural (NS) proteins NS-1 and NS-2 downregulate essential signalling molecules of the IFN induction and response pathways[31].

NOD2

IFN-β production by RSV-infected mouse alveolar macrophages, bone marrow-derived macrophages, and mouse embryonic fibroblasts depends on the cytosolic PRR NOD2[32]. Upon recognition of single-stranded viral RNA, NOD2 activates interferon-regulatory factor 3 (IRF3), leading to IFN-β production. NOD2-deficient mice infected with RSV fail to efficiently produce IFN-β and show exacerbated lung pathology[32]. Neutrophils express NOD2[15]; infection of neutrophils by RSV, resulting in cytosolic viral RNA[29], may thus result in detection of the virus by NOD2.

PRR-mediated detection of RSV

Taken together, neutrophils express multiple PRRs capable of sensing RSV, namely TLR4, TLR2/6 heterodimer, TLR7/TLR8, RIG-I, MDA5, and NOD2, and are thus well equipped to detect invading viruses. However, direct recognition of RSV by neutrophils via most of these receptors has yet to be confirmed experimentally. Nonetheless, the association of impaired TLR4 expression on neutrophils with severe RSV bronchiolitis suggests that neutrophil-mediated detection of RSV, via TLR4 at least, is important for an effective immune response against RSV.

Control of viral replication by neutrophils

Neutrophils mediate direct antimicrobial effects by, in essence, three known effector mechanisms, namely (i) phagocytosis, (ii) degranulation, and (iii), a more recently identified mechanism, neutrophil extracellular trap (NET) formation or NETosis[9]. See Table 1 for an overview of direct viral control effected by neutrophils.

Phagocytosis

By phagocytosis, neutrophils may eliminate RSV-infected cells, thereby preventing further viral replication, and engulf virions to limit infection of new cells. Support for this notion comes from experiments performed using influenza virus[33,34]. Firstly, pharmacological inhibition of phagocytosis increases mortality among influenza virus-infected mice[34]. Further, as visualised by microscopy, mouse neutrophils and macrophages both phagocytose influenza virus-infected, apoptotic cells *in vivo*. TLR4 signalling enhances this phagocytic activity[33]. Moreover, as shown *in vitro* for herpes simplex virus (HSV), antibody- and complement-mediated opsonisation facilitates the phagocytosis of virus particles and virally infected cells by neutrophils[35]. Thus, it is conceivable that neutrophil-mediated phagocytosis contributes to the elimination of RSV, which is similarly sensed by TLR4 and opsonised by antibodies.

Degranulation

Neutrophil granules are filled to the brim with antimicrobial proteins, including, *inter alia*, α-defensins 1-4 (or human neutrophil peptides [HNPs] 1-4), cathelicidins (i.e. LL-37), pentraxin (PTX)3, and myeloperoxidase (MPO), which are released into the microenvironment upon degranulation[9]. HNPs are small, cationic, amphipathic peptides with demonstrated antiviral activity. For instance, multiple steps of cellular entry (into CD4-, CCR5-, and CXCR4-expressing HeLa cells) by human immunodeficiency virus (HIV)-1 are inhibited by HNP-1[36], and HNPs promote influenza virus aggregation and uptake by neutrophils[37]. Whether the antiviral activity of HNPs extents to RSV is not currently known, but the broad antiviral activities of HPNs against multiple enveloped and non-enveloped viruses hints at the possibility[38]. Further, the RSV lipid envelope is sensitive to epithelial cell-expressed β-defensins-induced disintegration[39].

Via proteolytic processing, LL-37 is generated from its inactive precursor, human cathelicidin antimicrobial peptide of 18 kDa (hCAP-18), the sole member of the cathelicidin class of cationic antimicrobial peptides in humans. Recent *in vitro* work demonstrates that LL-37 possesses direct antiviral activity against RSV virions, protects infected epithelial cells against RSV-induced cell death, inhibits the

production of new virions, and reduces susceptibly of epithelial cells to RSV infection[40]. The clinical relevance of these results is supported by the finding that low serum levels of the LL-37 precursor, i.e. hCAP-18, are associated with severe paediatric RSV bronchiolitis, but not rhinovirus infection-induced bronchiolitis[41]. In addition, LL-37 was found to modulate TLR signalling. For instance, by binding to single- and double-stranded RNA, LL-37 enhances signalling via TLR7/TLR8 and TLR3, respectively[42,43].

Pentraxins are soluble pattern recognition molecules (PRMs) that serve as mediators of innate humoral immunity. PTX3 can activate compliment via the recruitment of C1q (i.e. the classical pathway) and act as opsonin by interacting with the FcγRIII (CD16) and FcγRII (CD32) receptors, which are expressed by phagocytes, including neutrophils[50]. Mature neutrophils represent a major reservoir of preformed, ready-to-use PTX3, as well as other PRMs[10]. Direct evidence of PTX3-mediated antiviral activity against RSV is lacking, but has been demonstrated for influenza virus and murine coronavirus (an animal model for severe acute respiratory syndrome [SARS])[44,45]. PTX3 binds influenza virus *in vitro* and impairs hemagglutination, infectivity, and neuraminidase activity. PTX3-deficient mice present with increased viral replication in lungs; the treatment of influenza virus-infected wild type mice with human PTX3 significantly improves survival and reduces viral load[44]. Simila rly, PTX3 binds to murine hepatitis virus strain 1 (MHV-1) coronavirus and reduces virus infectivity. MHV-1 infection-induced morbidity is greater in PTX3-deficient mice, and lung damage is attenuated by the administration of recombinant PTX3[45].

MPO catalyses the production of oxidative hypochlorous acid (HOCl) by utilising hydrogen peroxide (H_2O_2) generated by neutrophil NADPH-oxidase and local chloride ions (Cl^-). HOCl is a potent antimicrobial ROS, because of its ability to chlorinate and oxidise a great variety of biomolecules. The direct antiviral activity of MPO-produced HOCl against HIV-1 has long since been known[46]. A recent report suggests that the antiviral activity of MPO against HIV-1 may have an additional, indirect mode of action by altering serum proteins[47]. Amyloid-like HOCl-altered plasma proteins, including serum albumin, bind to HIV-1 particles and cellular proteins, resulting in reduced infectivity and viral replication *in vitro*. Although direct experimental support is lacking, it is feasible that HOCl and HOCl-modified proteins possess similar antiviral activity against RSV, because of the non-specific nature of the HOCl-induced molecular modifications. Further, in HSV-1 infection, an essential role for intracellular ROS in the stimulation of

Table 1. *Antiviral activities of neutrophils*. Neutrophil effector mechanisms and neutrophil-derived molecules and their respective antiviral activities are listed. The supporting lines of evidence and related references are included.

Mechanism or molecular mediator	Effect and function	Virus & Model	Reference
Phagocytosis	Eliminates virally infected cells	Influenza virus *In vivo*	33,34
HNP-1	Inhibits cellular entry	HIV *In vitro*	36
HNP-1 & HNP-2	Promotes virion aggregation & phagocytosis	Influenza *In vitro*	37
LL-37	Inactivates virions, protects epithelial cells from infection & cell death, inhibits virion production	RSV *In vitro* & clinical association	40,41
PTX3	Reduces virion infectivity	Influenza virus & MHV-1 *In vitro* & *in vivo*	44,45
MPO	Inactivates virions & generates antiviral proteins by chemical modification via HClO production	HIV-1 *In vitro*	46,47
NETosis	Reduces viral infectivity & spread, inactivates virions	Influenza virus & myxoma poxvirus *In vitro* & *in vivo*	48,49

the innate anti-viral immune response was demonstrated[51]. Intracellular ROS are also required for inflammasome assembly during RSV infection[52]. In RSV infection, oxidative stress, partially induced by viral inhibition of antioxidant enzymes, was also contributes to the lung pathology of severe bronchiolitis[53,54].

NETosis

Two recent studies support a role for NETs in antiviral defence[48,49]. NETs are genomic DNA-based meshes decorated with antimicrobial proteins, including MPO and α-defensins. By trapping microbes in NETs, mediated in part by NET-bound PRMs such as PTX3, microbial spread can be minimised and microbe killing optimised by high local concentrations of antimicrobial proteins[55]. TLR7/TLR8-mediated detection of HIV-1 by neutrophils was shown to trigger NADH-oxidase-dependent ROS production, which induces NETosis. Thus formed MPO- and α-defensin-rich NETs trap HIV-1 virions *in vitro*, an interaction that is mediated by the negatively charged viral envelope and positively charged NET-bound histones. By using MPO inhibitor and anti-α-defensin neutralizing antibody, NET-bound MPO and α-defensins were shown to abolish viral infectivity. In co-culture, neutrophils impair HIV-1 infection of CD4+ T cells in an extracellular DNA-dependent manner, as shown by addition of DNAse to the culture medium. As a countermeasure, HIV-1 induces IL-10 secretion by DCs, which prevents NET formation[49].

In the second study, the systemic administration of viral analogues (i.e. viral PAMP-imitating TLR agonists), as well as myxoma poxvirus infection, induced the recruitment of neutrophils to the liver sinusoids in mice. There, interactions between aggregated platelets and neutrophils trigger the release of NETs. When poxvirus-inoculated mice are treated intravenously with DNAse I, more liver cells become infected. This study thus demonstrates, *in vivo*, the protective role of NETs in viral infection[48].

An earlier study on respiratory influenza infection suggests that excessive NET formation contributes to the immunopathology, rather than aid viral clearance or impair viral infectivity[56]. Extensive NETosis is observed in the lungs, in particular in the alveoli, of mice challenged with lethal doses of influenza virus. Co-culture of mouse neutrophils with influenza virus-infected epithelial cells, but not uninfected cells, strongly induces NET formation in an MPO-dependent fashion. Culturing mouse neutrophils in the presence of H_2O_2 induces NETosis and damages human umbilical vein endothelial cells (HUVECs). This suggests that NETs mediate endothelial tissue damage during influenza virus infection[56]. NET-induced tissue damage during influenza virus infection was also examined in mice unable to form NETs[57]. The peptidylarginine deiminase (PAD)4-mediated deimination of H3 and H4 histones is required for NETosis, hence PAD4-deficient mice do not form NETs[58]. PAD4-deficient mice challenged with influenza virus perform similar to wild type mice. No significant differences are observed in leukocyte recruitment to the lungs, cytokine levels, or viral loads. This report thus suggests no notable *in vivo* contribution, protective or pathological, of NETs to the pathogenesis of respiratory influenza virus infection[57].

In addition, NETs can trigger IFN-α production by plasmacytoid DCs (pDCs) via the TLR9 pathway. Chronic NET-induced IFN-α release has recently been proposed to be at the core of systemic lupus erythematosus (SLE) pathogenesis[59,60]. Nonetheless, IFN-α mediates potent antiviral effects. In viral infection therefore, induction of IFN-α secretion by pDCs may represent an indirect contribution of NETs to antiviral defence.

The role of NETs in the pathogenesis of RSV infection has yet to be studied. However, NETs clearly possess direct, and possibly indirect, antiviral activity. While the possibility that excessive NET formation may induce collateral tissue damage remains, especially in sensitive airways, NETosis appears to be a functional neutrophil response to limit viral infection. Together with phagocytosis and degranulation, NETosis thus mediates direct antiviral activity of neutrophils.

Neutrophil cellular cross-talk

Recent studies demonstrate multiple immune regulatory functions for neutrophils. Therefore, in addition to their role as effector cells in RSV infection described above, neutrophils may also act as immune regulatory cells. Although immune regulation by neutrophils in the context of RSV infection has yet to be examined directly, general concepts may be gleaned from these studies and guide further research into the role of neutrophils in RSV infection. Figure 1 summarises neutrophil-mediated cellular crosstalk.

Neutrophil-mediated stimulation of T cell responses

Mouse neutrophils were recently demonstrated to differentiate into neutrophil-DC hybrid cells with combined professional phagocyte and antigen-presenting cell (APC) functionality[61,62]. While retaining distinct neutrophil capabilities such as NETosis and cathelicidin-mediated killing of phagocytosed bacteria, neutrophil-DC hybrid cells acquired expression of TLR3 and co-stimulatory CD40, copiously produced cytokines upon TLR stimulation, and stimulate the proliferation of naïve CD4+ T cells. Thus-activated CD4+ T cells produce IFN-γ and IL-17, indicative of Th1/17 polarisation. However, cross-presentation of antigen to CD8+ T cells is limited[62]. Inflammation induces the differentiation of mouse neutrophils into neutrophil-DC hybrid cells *in vivo*. Depletion and reconstitution experiments in mice show that neutrophil-DC hybrid cells contribute to bacterial clearance and the induction of CD4+ T cell responses *in vivo*[61]. Thus, neutrophils can differentiate into potent APCs that retain neutrophil-specific antimicrobial qualities and induce Th1/17-skewed CD4+ T cell responses[61,62]. Further research should investigate the role of neutrophil-DC hybrid cells in antiviral defence.

While the neutrophil-DC hybrids appear primarily involved in the induction of CD4+ T cell responses, other recent reports suggest a role for neutrophils in promoting CD8+ T cell responses[63-65]. Influenza virus-infected neutrophils present viral antigen to CD8+ T cells *in vitro* and thereby stimulate IFN-γ production. Further, the amount of IFN-γ and IFN-γ-producing CD8+ T cells in the lungs is decreased in neutrophil-depleted mice[64]. Virally infected epithelial cells, however, elicit cytolysis by CD8+ T cells, but not IFN-γ production[66]. Neutrophils were thus able to regulate CD8+ T cell effector function at the site of viral infection[64,66]. Whether neutrophils play a similar role during RSV infection remains to be determined. However, RSV-infected neutrophils express viral proteins[29], thereby lending credence to the possibility.

Furthermore, non-infected neutrophils are reported to efficiently cross-prime naïve CD8+ T cells *in vivo*[65]. Both human and mouse antigen-pulsed neutrophils trigger CD8+ T cell activation *in vitro*. Cross-presentation to naïve CD8+ T cells by neutrophils *in vivo* is demonstrated using β2-microglobulin-deficient mice that lack surface expression of MHC class I molecules, and therefore possess no cells capable of antigen presentation to CD8+ T cells. *Ex vivo* antigen-pulsed neutrophils and antigen-specific CFSE-labelled CD8+ T cells were transferred into β2-microglobulin-deficient mice. Proliferated CD8+ T cells, measured by CFSE dilution, were observed in the lymph nodes and spleen. These CD8+ T cells acquire the capability to lyse peptide-pulsed cells and secrete IL-2 and IFN-γ *ex vivo*[65]. Whether neutrophils cross-present RSV antigen is not known. Nonetheless, neutrophils are reported to engulf influenza virus-infected, apoptotic cells[33], and may similarly phagocytose RSV-infected cells to acquire viral proteins for cross-presentation.

Neutrophils may also act as intermediaries in the induction of CD8+ T cell responses by transporting viral antigen. Neutrophils take up modified vaccinia Ankara (MVA) virus in skin, transport viral antigen to the bone marrow, and there, via local APCs, trigger CD8+ T cell proliferation[63]. Live imaging reveals neutrophil-mediated transport of modified vaccinia Ankara (MVA) virus from the dermis to the bone marrow. Intradermal injection of MVA virus or *ex vivo* MVA virus-loaded neutrophils induces proliferation

among bone marrow CD8+ T cells, whereas such proliferation is abrogated in neutrophil-depleted mice injected with MVA virus. The response is similarly abolished if bone marrow phagocytic APCs are depleted with clodronate-containing liposomes. Virus-carrying neutrophils are suggested to be phagocytosed by APCs in the bone marrow, which cross-present antigen to induce an antiviral CD8+ T cell response. Although there are no reports on neutrophil-mediated transport of other viruses from other organs, it is conceivable that neutrophils may mediate the transport of RSV antigen from the lungs to the bone marrow.

Recently, RSV was shown to infect human bone marrow stromal cells (BMSCs) *in vitro* and viral RNA was detected in human naïve primary BMSCs[67]. The discovery that RSV infects neutrophils[29], and that neutrophils transport virus to bone marrow[63], hints at the possibility that neutrophils may also serve as a Trojan horse for RSV by providing transport to an extrapulmonary target[67].

Collectively, these studies unveil the capability of neutrophils to induce and polarise T cell responses. Thereby, neutrophils can contribute indirectly to the control of viral replication. The physiological relevance of neutrophil-induced T-cell responses in viral infections in general, and RSV infection in particular, invites further research.

Neutrophil-mediated suppression of T cell responses

In addition to the stimulation of T cells by neutrophils described above, recent work demonstrates that a subset of systemic inflammation-induced human neutrophils is able to suppress T cell responses[68]. The intravenous injection of LPS into healthy volunteers triggers systemic inflammation with pronounced neutrophilia. This was found to include the appearance of a phenotypically and morphologically distinct neutrophil subset into circulation. In co-culture with peripheral blood mononuclear cells (PBMCs), this neutrophil subset, but not others, suppress T cell proliferation in the presence of T cell stimulatory agents. Cellular proximity is required for T cell suppression, and further depends on neutrophil Mac-1 and H_2O_2 production, but not on profuse H_2O_2 release into the extracellular environment. Rather, real-time imaging reveals transient interactions between neutrophils and T cells, during which bursts of neutrophil-membrane associated H_2O_2 are generated in the immunological synapse. In the presence of blocking anti-Mac-1 antibody, the frequency of interactions is notably reduced. The suppressive neutrophil phenotype could not be induced *ex vivo* in response to pro- or anti-inflammatory stimuli[68]. Whether the suppressive neutrophils identified in this study reflect the plasticity of *in vivo* circulating neutrophils, or the recruitment of suppressor neutrophils to the circulation from tissue(s) during systemic inflammation remains to be definitively determined.

Further immune suppressive abilities of neutrophils are described in a recent study which demonstrates that neutrophils limit the magnitude and spread of T cell responses[69]. Depletion of neutrophils by anti-Ly6G antibody treatment prior to immunisation with protein in adjuvant enhances the total T cell response in local and distal lymph nodes, as measured by cytokine production. Moreover, in regular mice, approximately 25% of the total T cell response is found in the distal lymph nodes, compared to 70% in the absence of neutrophils. Enzyme-deficient mice and pharmacological inhibitors were used to identify that neutrophil-produced prostanoids, specifically thromboxane, mediate the suppression. An earlier study had demonstrated that neutrophil entry into the lymph node reduces the frequency and duration of contacts between DCs and T cells[70]. At the time, a molecular mechanism was not identified, but the subsequent study suggests that it is mediated by neutrophil-derived thromboxane[69]. Indeed, thromboxane A_2 is reported to modulate CD4+ T cell responses by inducing random T cell movement that impair DC-T cell interactions[71]. Moreover, thromboxane induces lymphatic vessel contraction[72,73], and thereby may impair cellular migration from local to distal lymph nodes.

Infection-induced immunity to RSV is of short duration and incomplete; reinfections occur frequently[74]. Whether neutrophil-mediated T cell suppression plays a role herein, or whether it prevents excessive T cell-mediated damage to the airways is not known. The contribution of neutrophil-mediated T cell suppression to the control of viral infections awaits elucidation.

Neutrophil-induced antibody production

Recent work demonstrates that neutrophils promote the production of 'innate antibodies'[75]. Spleen marginal zone (MZ) B cells represent a unique B cell population that does not require T cell-dependent germinal centre reactions. Positioned in the peri-MZ areas, between MZ B cells and sinusoidal endothelial cells (SECs), neutrophils are found in large numbers in the spleens of people, rhesus macaques, and mice under non-inflamed conditions. In co-culture, splenic neutrophil-derived BAFF, APRIL, and IL-21 stimulate antibody production, class-switch recombination, and somatic hypermutation in MZ B cells. The levels of IgA, IgG, and IgM antibodies against T cell-independent antigens, but not T cell-dependent antigens, are notably reduced in neutropenic patients. Circulating neutrophils co-cultured with LPS-activated SECs acquire a splenic neutrophil phenotype, capable of stimulating MZ B cells. Thus, upon reprogramming in the spleen, neutrophils stimulate the MZ B cell antibody response against T cell-independent antigens via secretion of BAFF, APRIL, and IL-21[75]. Whether such antibodies provide protection against RSV-induced disease remains to be determined. Nonetheless, RSV-specific antibodies are protective against severe RSV bronchiolitis, as evidenced by the efficacy of RSV-specific antibody prophylaxis in high-risk infants [76,77]. Moreover, there are precedents for protection against viral infection by T cell-independent antibodies[78,79].

Neutrophil-mediated regulation of DCs

The regulation of adaptive immune responses by neutrophils is not limited to direct effects on lymphocytes, but extends to neutrophil-mediated modulation of DC function at multiple levels. In *Leishmania major* infection of mice, neutrophil-secreted CCL3 is critical to the recruitment of immature DCs to the site of parasite inoculation[80]. Through neutrophil-specific glycosylation, Mac-1 and CAECAM1 on activated, but not resting, neutrophils interact with DC-SIGN to trigger DC maturation and subsequent DC-induced proliferation and Th1 polarisation of T cells[81,82]. Frequent DC-neutrophil contacts are observed *in situ* in the inflamed intestinal tissue of Crohn's disease patients[82]. In mouse lymph nodes, neutrophils were found to deposit MPO via degranulation, where it impaired DC function[83]. MPO suppressed the activation, migration to lymph nodes, and antigen uptake of mouse DCs *in vivo*. In culture, neutrophil-derived MPO also impaired the activation of human DCs[83]. Thus, similar as for T cells, neutrophils can both stimulate and inhibit DC function. How this relates to viral infection at large, or RSV infection in particular, has yet to be examined.

Neutrophils regulate NK cell functionality and homeostasis

Natural killer (NK) cells are innate cytotoxic lymphocytes that protect against virus-infected and tumour cells. Recent work has provided compelling evidence for neutrophils as regulators of NK cell and function and homeostasis[84-86]. In Gfi-1-mutated mice with defective terminal differentiation of neutrophils, NK cells are hyporesponsive. Moreover, wild type NK cells transferred into Gfi-1-mutated mice become hyporesponsive and NK cells from Gfi-1-mutated mice become responsiveness in wild-type mice. Neutrophil depletion by anti-Ly6G antibody treatment of wild-type mice reduces NK cell functional responsiveness and survival. These results were confirmed in neutropenic patients, whose NK cells show a more immature phenotype with reduced functional responsiveness[84]. Direct contacts between NK cells and neutrophils are observed in mouse lymphoid organs[84] and inflamed human tissues[85]. Mouse neutrophils directly stimulate NK cells to produce IFN-γ by secreting IL-18[86]. Nonetheless, an intermediate cell type via which neutrophils

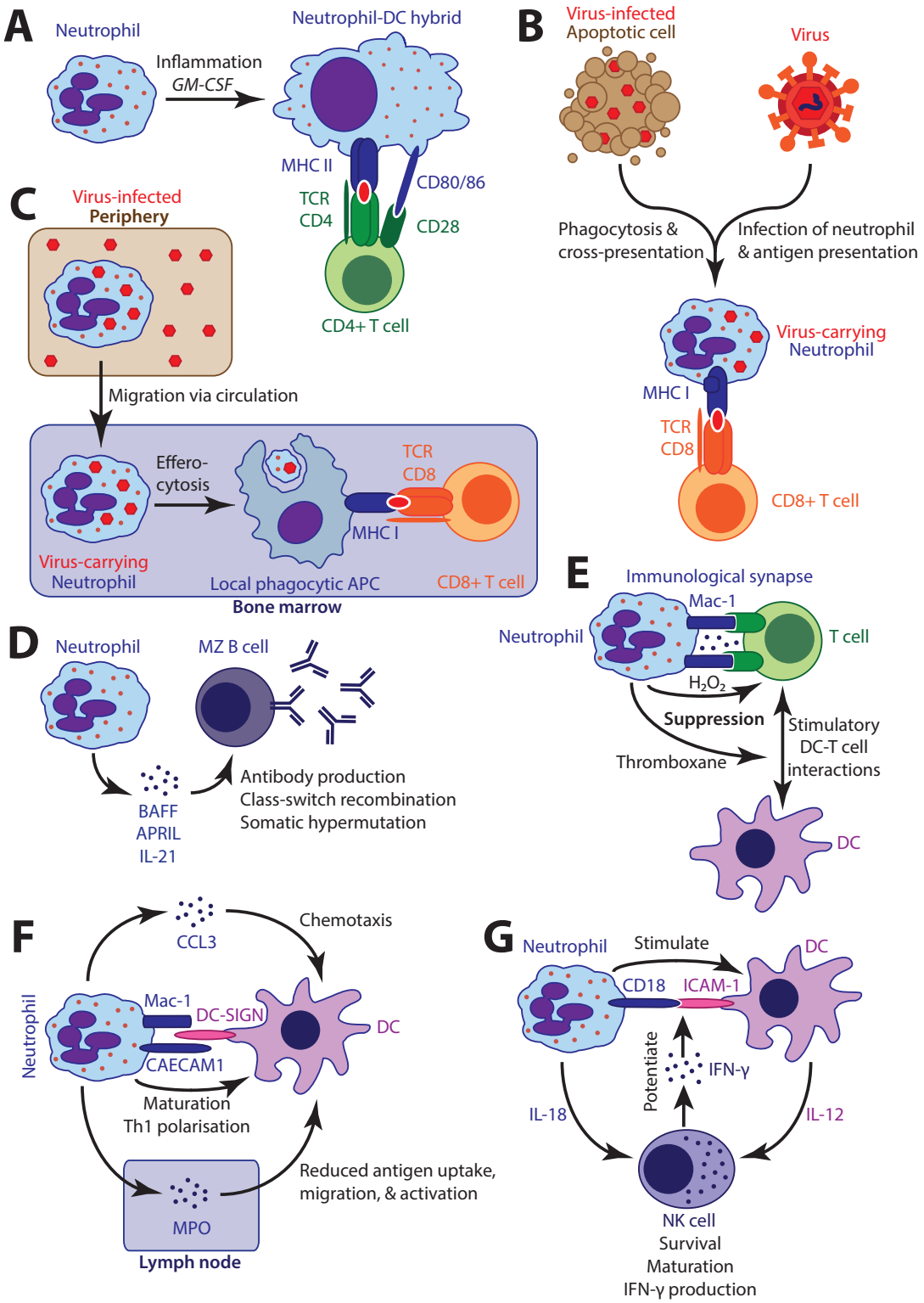

Figure 1. *Neutrophil cellular crosstalk*. This figure provides a visual overview of recently identified cellular crosstalk among immune cells that involves neutrophils. For in-depth textual descriptions, see the main text and the therein-referenced reports. Neutrophils directly stimulate both CD4+ T cells (**A**) and CD8+ T cells (**B**), and CD8+ T cells indirectly by antigen transport (**C**). Antibody production, class-switch recombination, and somatic hypermutation are triggered in MZ B cells by neutrophil-secreted factors (**D**). Via H_2O_2 released in a Mac-1-mediated immunological synapse and the secretion of thromboxane, neutrophils inhibit T cells directly and by interrupting DC-T cell interactions, respectively (**E**). Neutrophils attract DCs with CCL3 and stimulate maturation and Th1 polarisation by direct interaction, but can also reduce DC antigen uptake, migration and activation by secreting MPO (**F**). In collaboration with DCs, neutrophils sustain NK cell homeostasis; an interaction that NK cells potentiate by induced IFN-γ secretion (**G**).

effect their stimulation on NK cells cannot be excluded. Indeed, a collaboration between neutrophils and DCs to stimulate IFN-γ secretion by NK cells, which in turn further potentiates the interaction between neutrophils and DCs, was recently identified[85].

However, whether neutrophil-stimulated NK cells protect against RSV disease is uncertain. NK cell-depleted mice infected with RSV or influenza virus present with ameliorated, rather than exacerbated, lung pathology[87-89]. Therefore, in severe RSV bronchiolitis and influenza infection, NK cells may represent a pathological rather than protective immune cell infiltrate. Neutrophil-maintained NK cell homeostasis may thus not contribute to limit RSV replication, but instead may promote lung injury.

Apoptotic neutrophils trigger anti-inflammatory macrophage polarisation

Depending on environmental stimuli, macrophages can acquire a pro-inflammatory IL-12^{high} IL-10^{low} (classically activated or M1) or pro-resolution IL-12^{low} IL-10^{high} phenotype (alternatively activated or M2). The phagocytosis of apoptotic neutrophils by macrophages was found to induce a regulatory IL-12^{low} IL-10^{high} phenotype in macrophages, which may contribute to the resolution of inflammation[90]. In RSV-infected cotton rats, alternatively activated macrophages were found to control lung damage[91]. However, in this study, the contribution of neutrophil efferocytosis to macrophage polarisation was not examined.

Perspective: The Janus-faced nature of neutrophils in viral respiratory infection

A beneficial role for neutrophils in viral respiratory infection has recently been reported for influenza virus[92-94]. Neutrophil-depletion in mice demonstrates that neutrophils ameliorate influenza virus infection-induced lung injury. In the absence of neutrophils, an infection with otherwise only mildly virulent influenza virus results in severe disease[92]. The protective role of neutrophils in influenza infection was confirmed in another recent study that identified the critical contribution of IL-6 to the prevention of lung damage and subsequent death. IL-6 supresses influenza virus-induced apoptosis of neutrophils, without which the virus could not be cleared efficiently[93]. Moreover, depletion of mouse neutrophils by anti-Ly6G antibody treatment demonstrates that neutrophils limit the influenza virus infection-induced disease severity of intermediate and highly virulent strains, but are not essential to the control of mildly virulent influenza strains[95].

Nonetheless, impairment of CXCL2-mediated neutrophil infiltration into the lungs of influenza virus-infected mice reduces lung pathology, without affecting viral replication[96]. Further, while the release of matrix metalloprotease (MMP)9 is required for neutrophil migration to the airways and the control of viral replication, it also contributes to lung injury[8]. Excessive neutrophil recruitment may thus effect pathological consequences. Whether the direct antiviral activity of neutrophil effector mechanisms or neutrophil-mediated immune regulation is responsible for the neutrophil-mediated protection against influenza virus infection, and similarly for RSV infection, remains to be determined.

A newly developed attenuated *Bordetella pertussis* vaccine was recently tested in mice for potential interactions with subsequent RSV infection[97]. Enhanced induction of IL-17 and recruitment of neutrophils to RSV-infected lungs is observed in vaccinated mice, but without exacerbation of lung injury. To the contrary, prior *B. pertussis* vaccination provides notable protection against subsequent RSV infection in an IL-17-dependent manner, as confirmed by anti-IL-17 blocking antibodies[97]. Although neutrophil depletion was not examined, this study suggests that neutrophils are not necessarily pathological in RSV infection, and may even play a protective role. However, excessive IL-17-induced neutrophil recruitment to RSV-infected lungs is also suggested to contribute to lung pathology[98]. Indeed, the severity of paediatric RSV bronchiolitis correlates with the degree of pulmonary neutrophilic inflammation[99]. Further, a common single nucleotide polymorphism just upstream of the IL-8 gene, which is

associated with increased IL-8 production, a potent neutrophil chemoattractant, is more frequent among infants with bronchiolitis, in particular among infants who lack known risk factors[100]. Collectively, these studies highlight the Janus-faced nature of neutrophils during viral respiratory infection, to which both lung injury and protection therefrom are ascribed.

Conclusion

The role of neutrophils in RSV infection remains to be fully delineated. While neutrophils are certainly capable of instigating immunopathology, the newly identified regulatory functions of neutrophils and the antiviral activity of effector mechanisms suggest a multitude of ways in which neutrophils may contribute to the control of RSV infection. Degranulation releases antiviral mediators, phagocytosis eliminates virions and virally infected cells, and NETs capture and inactivate virus particles. Neutrophils activate, suppress, and polarise T cell responses, modulate DCs, promote antibody production, support NK cells, and induce pro-resolution macrophages. The first reports of neutrophil-mediated contributions to the control of viral infection have recently emerged. In severe respiratory influenza virus infection, neutrophils ameliorate lung pathology and reduce mortality.

Nonetheless, neutrophil-derived ROS, proteases, and NETs are equally cytotoxic to bystander cells, and thereby invariably induce collateral tissue damage. Indeed, the extent of neutrophilic airway inflammation and a genetic predisposition towards increased production of the neutrophil-recruiting chemokine IL-8 correlate with severity of disease in paediatric RSV bronchiolitis patients. Thus, neutrophil-mediated immunopathology and viral clearance appear to be two sides of the same coin, where a delicate balance determines whether it turns up heads or tails. The balance of evidence hangs against neutrophils as purely pathological cells in RSV infection.

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