Eosinophil Response Against Classical and Emerging Respiratory Viruses: COVID-19

Short running title: Eosinophils and respiratory viruses

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ABSTRACT

Eosinophils were discovered more than 140 years ago. This polymorphonuclear leukocyte has a very active metabolism, containing numerous intracellular secretory granules that allow it exerts multiple functions in both health and disease status.

Classically, eosinophils have been considered as important immune cells in the pathogenesis of inflammatory processes such as parasitic helminth infections and allergic or pulmonary diseases like asthma, being always associated to a type 2 immune response; furthermore, in the last years, it has been linked to immune response conferring host protection against fungi, bacteria, and viruses, recognizing them through several molecules such as toll-like receptors (TLRs) or retinoic acid-inducible gene 1 (RIG-1)-like receptor (RLR).

The immune protection is exerted through multiple mechanisms and properties of these cells. They contain numerous cytoplasmatic granules that release cationic proteins, cytokines, chemokines and other molecules that contribute to their functions.

In addition to their competence as effectors cells, its capabilities like antigen-presenting cell allow them to act in multiple situations promoting diverse aspects of the immune response.

This review summarizes diverse aspects of eosinophil biology and mainly, it goes over the mechanisms and roles carried out by eosinophils in host defence against virus infections and vaccines response, focusing the attention in respiratory viruses like the new coronavirus, SARS-CoV-2.

Key words: Eosinophils. Respiratory viruses. Immune response. Vaccines. Emerging viruses.

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RESUMEN

Los eosinófilos fueron descubiertos hace más de 140 años. Este leucocito polimorfonuclear tiene un metabolismo muy activo y contiene numerosos gránulos secretores intracelulares que le permiten ejercer múltiples funciones tanto en el estado no patológico como en el de la enfermedad. Clásicamente, los eosinófilos se han considerado como importantes células inmunes en la patogénesis de procesos inflamatorios tales como infecciones parasitarias por helmintos y enfermedades alérgicas y/o pulmonares como el asma, las cuales están asociadas a una respuesta inmune tipo 2. Además, en los últimos años, los eosinófilos también han sido relacionados con la respuesta inmunológica que confiere protección al huésped contra hongos, bacterias y virus, reconociéndolos a través de varias moléculas como los receptores tipo Toll (TLR) o los receptores parecidos al gen inducible por ácido retinoico 1 (RIG-1) o RLR.

La protección inmune es ejercida a través de los múltiples mecanismos y propiedades características de estas células. Contienen numerosos gránulos citoplasmáticos que liberan proteínas catiónicas, citocinas, quimiocinas y otras moléculas que contribuyen a estas funciones. Además de su competencia como células efectoras, sus capacidades como célula presentadora de antígeno les permite actuar en múltiples situaciones, promoviendo diversos aspectos de la respuesta inmune.

En esta revisión se resumen diversos aspectos de la biología de los eosinófilos y, principalmente, se repasan los mecanismos y funciones que desempeñan estas células en la defensa del huésped contra las infecciones por virus, así como la respuesta desencadenada por las vacunas víricas, focalizando la atención en los virus respiratorios como el nuevo coronavirus SARS-CoV-2.

Palabras clave: Eosinófilos. Virus respiratorios. Respuesta inmune. Vacunas. Virus emergentes. COVID-19.

1. INTRODUCTION

Due to the current COVID-19 pandemic, all efforts are currently focused towards understanding this new infectious disease, specifically in unravelling the pathophysiological mechanisms for helping in vaccine development. The aim of this review is to describe the current knowledge of eosinophil roles against viruses, and how they are implicated in vaccine responses; focusing on getting an insight into how eosinophils affect and are affected by SARS-CoV-2 in COVID-19.

1.1. The eosinophil: a versatile cell.

Eosinophils were described for first time by Paul Ehrlich in 1879, identifying them using aniline dye eosin [1]. These cells can be easily differentiated from others, such as neutrophils and basophils, based on their morphology characteristics and brightly brick-red appearance when stained with haematoxylin and eosin [2]. Since many years ago, the eosinophil has been object of extensive investigation but, the role of this cell in health and disease remains controversial and imprecisely defined [3].

Commonly, eosinophils are a kind of white blood cells with bilobed nucleus. They are polymorphonuclear leukocytes that lack of proliferative capacity, with a lifespan between 8 to 12 hours before they migrate into tissues, where they can survive several days [4]. Normally, they circulate in the blood stream in a low percentage, assuming 3 to 6% of the total granulocytes. Also, these cells have a very active metabolism and they are characterized by containing numerous intracellular secretory granules in the cytoplasm [5].

Eosinophils are produced in the bone marrow from pluripotential stem cells, that differentiate towards an independent eosinophil lineage [6]. Eosinophil lineage specification is dictated by the

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interplay of at least three classes of transcription factors, including GATA-1 (a zinc family finger member), PU.1 (an ETS family member), and C/EBP members (CCAAT/enhancer-binding protein family) [7]. It is important to note that three cytokines are particularly important in regulating eosinophil development: interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These eosinophilopoietins likely provide permissive proliferative and differentiation signals following the instructive signals specified by the above mentioned transcription factors, being IL-5 the most specific to the eosinophil lineage [8]. Also, this cytokine is responsible for selective differentiation of eosinophils and stimulates the release of these leukocytes from the bone marrow into the peripheral blood [9] .

Eosinophils contain in their specific granules preformed arsenal of cationic granule proteins, cytokines, chemokines, growth factors, lipid mediators, and other immunomodulatory molecules, including matrix metalloproteinases, which will serve the eosinophils to exercise their functions [10]. The main eosinophil-derived cationic granule proteins are major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) which will be important elements in the functionality of eosinophils [11].

1.2. Eosinophil role in immune response against helminth, fungi, bacteria, and viruses

Eosinophils are multifunctional leukocytes. Historically, they have been considered as important immune cells in the pathogenesis of numerous inflammatory processes, including parasitic helminth infections and allergic diseases, such as asthma [12–14]. In response to diverse stimuli, eosinophils are recruited from the blood circulation into the inflammatory focus, where they

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modulate immune responses through releasing an array of cytokines and other mediators, as well as by a broad spectrum of immune mechanisms [15].

Triggering of eosinophils by engagement of receptors for cytokines, immunoglobulins, and complement can lead to the secretion of pro-inflammatory cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, transforming growth factor (TGF)-α/β), chemokines (CCL5 and eotaxin-1(CCL11)), or lipid mediators (platelet-activating factor (PAF) and leukotriene C4 (LTC4)) [16]. Eosinophils cause tissue damage, by releasing of plethora of toxic proteins and other preformed pro-inflammatory mediators contained in their granules through degranulation processes [17].

Eosinophils can actively promote type 2 immune responses by producing a range of immunoregulating cytokines and other factors [18]. Also, they can function as non-professional antigen-presenting cells (APCs), processing and presenting a variety of microbial, viral, and parasitic antigens [19]. This cell type in resting state do not constitutively express MHC class II molecules or co-stimulatory molecules on the surface, but MHC class II molecules can be expressed on eosinophils upon activation by some cytokines [20].

Eosinophils have been considered as end-stage cells in innate immunity contributing to anti-parasitic immunity or allergy by their pro-inflammatory and destructive effects. In the last decades, many new roles of eosinophils have been identified in various pathological processes, including host protection against other pathogens such as certain types of fungi, bacteria, and viruses [17]. Khan et al reported high levels of type 2 interleukins in human patients with basidiobolomycosis [21]. These findings support other results that indicate that during *Basidiobolus* and *Conidiobolus* infections a type 2 immune response is likely activated, increasing blood counts of eosinophils in patients infected with these fungi [22]. Eosinophils

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have been studied as a marker of bacterial infections, since animal studies showed that peripheral eosinophil count decreased with acute bacterial infection, because of the accumulation of eosinophils at the inflammatory site and inhibition of egress from the bone marrow [23,24]. Moreover, evidences are emerging that eosinophils may also have a protective role in viral infections, especially against RNA viruses such as respiratory syncytial virus (RSV) and the influenza virus [25,26].

All mentioned characteristics make eosinophils very versatile and functional cells with a lot of resources in host defence.

2. THE EOSINOPHIL IMMUNE RESPONSE AGAINST VIRUSES

2.1. Mechanisms for eosinophil viral recognition and clearance

Eosinophils can recognize and react against one of the smallest of pathogens: viruses. Viruses have in common a genome inside a protein capsid, which in some cases is protected by a lipid bilayer. There is controversy regarding if viruses are alive or non-alive entities, however everybody agrees that viruses are dependent on infecting hosts to replicate [27].

2.1.1. Recognition of virus by eosinophil receptors

The recognition of viral particles is made by pattern recognition receptors (PRRs), among those including Toll-like receptors (TLRs), and retinoic acid-inducible gene 1 (RIG-1)-like receptor (RLR) which are able to identify pathogen-associated molecular patterns (PAMPs), molecules that are characteristic of different pathogens. The TLRs can enable signalling pathways that

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orchestrate antigen specific immune responses. In humans there are ten different TLRs, each of one being able to recognize distinct PAMPs [28] (Figure 1).

Eosinophils express several TLRs, such as TLR1, TLR3, TLR4, TLR7, TLR9, and TLR10 [29].TLR7 is one of the most important viral receptors in eosinophils, as the expression of this receptor, which recognizes single stranded RNA (ssRNA), is higher in these cells compared to neutrophils, and signalling through it increases the expression of adhesion molecules in eosinophils like L-selectin and CD11b, induces the generation of superoxide anions and promotes survival after activation by IFNγ [26,30,31].

These results were further confirmed for both TLR7 and TLR9 in eosinophils, with an increase in the secretion of IL-8 and enhanced survival, chemotactic migration (CD11b^{High}/L-selectin^{Low}), elevated activation (CD69) and EDN secretion after stimulation of the receptors with agonists. Besides, priming eosinophils with histamine, IL-4 and IL-5 caused even higher responses for TLR7 and TLR9 activation as seen in increased EDN and IL-8 secretion [32]. TLR7 signalling pathway seems to be dependent on prolyl isomerase Pin1, being this molecule a key factor in the antiviral response of eosinophils and in eosinophil generation in bone marrow, in an IRAK4 dependent process [33].

Some of these receptors have been associated to allergic diseases, as TLR3, which is a double stranded RNA receptor which is decreased in eosinophils in allergic rhinitis season and by type 2 cytokines, providing a possible link between viral infection and allergic exacerbations [34,35].

Also, eosinophils express RIG-1 receptor, which recognizes RNA sequences with 5’ triphosphorylated ends, although expression of this receptor is lower in eosinophils compared to

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neutrophils, and agonist stimulation does not cause any effect, meaning that this receptor may be inactive [36,37].

Not only eosinophils are able to recognize viral particles. They are also capable to recognize products of cell necrosis; as they express HMGB1 receptor, which induces eosinophil degranulation, being this an alternative pathway to respond to tissue damage by virus like influenza [38,39].

2.1.2. Eosinophils can perform antigen presentation

Eosinophils express molecules involved in antigen presentation as a mechanism for immune induction. Treatment with GM-CSF induces eosinophils' expression of MHC-II molecules and the co-stimulatory proteins CD40, CD80, and CD86, being able to present the antigen ovalbumin to $CD4^+$ lymphocytes inducing their proliferation and expression of IL-4 and IL-2; or to present parasitic antigens, eliciting IL-5 secretion by lymphocytes [19,40–42]. Also, eosinophils express CD28, and ligation of this molecule, even independently of other signals, increases the secretion of IL-2 and IFNγ by these cells [43].

Eosinophils antigen presentation is also vital in the immune mechanisms against viral infections. During the 2009 influenza pandemic the asthmatics were more likely hospitalized, but they presented less severity status disease and mortality. Using mouse models, Samarasinghe et al showed that eosinophils from lungs of allergic asthmatic mice were activated by influenza virus, enhancing piecemeal degranulation and upregulating antigen presentation markers. Eosinophils were able to migrate to the draining lymph nodes and present viral antigens to $CD8^+$ T cells, resulting in activation and proliferation of these cells [44]. Other study showed that eosinophils expressing intercellular adhesion molecule (ICAM)-1 after GM-CSF treatment are able to

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present antigens from rhinoviruses to T cells promoting their proliferation and IFNγ secretion [45].

Depending on the antigen type, eosinophils will be able to induce the activation and proliferation of $CD8^+$ or $CD4^+$ T cells. Eosinophils are able to migrate from the endobronchial lumen, to the paratracheal draining lymph nodes or thoracic lymph nodes to stimulate $CD4^+$ T cells proliferation by antigen presentation [46,47]. This migration does not seem dependent on eotaxin, but instead depended on CD80 and CD86 co-stimulation, being both molecules activated by cytokines like IL-3, and with key roles in antigen presentation of eosinophils, which induces proliferation of T lymphocytes in thymus and lymph nodes [46,48,49]. Despite being antigen presenting cells, eosinophils do not seem to be as effective as monocytes or dendritic cells; nonetheless, their action must not be disregarded [50].

The expression of specific receptors against pathogens, and co-stimulatory molecules, summed to their capacity to migrate to regional lymph nodes makes eosinophils cells profoundly implicated in immune responses against viruses, with the ability to act as antigen presenting cells [20].

2.1.3. Antiviral action of activated eosinophils in the immune response

Another set of eosinophils' mechanisms against viruses is probably one of the most important, as it gives them the capacity to perform direct action against viruses. This is based on their capacity to synthesise molecules, which are stored inside cytoplasmic granules and their releasing generate damaging effects. Among these molecules are ECP and EDN. These molecules have a main role as anti-parasitic and anti-bacterial molecules when released from granules [11]. Nevertheless, they are also effective against viruses due to their ribonuclease (RNase) activity, as

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both EDN and ECP have been shown to destruct the extracellular virions of RSV group B suspensions, and EDN has even been described to have effect against hepatitis B virus [51–56]. Indeed, eosinophils might be one of the first lines of defence against virus. The infection of mice with pneumovirus causes an elevation of eosinophils in the airways at day three, which associates with increased levels of the eosinophil chemoattractant macrophage inflammatory protein-1-alpha (MIP-1α) [57]. Eosinophils are critical in the defence against mice pneumovirus, as they are needed to perform antiviral clearance and promote survival against lethal infection when activated in Th2 environment [58].

Another principal characteristic of eosinophilic host defence is their capacity to synthesise and release compounds derived from oxygen or nitrogen. Through enzymes like EPO or inducible nitric oxide (NO) synthase (iNOS) they are able to produce both NO and bromide derived oxidant agents with damaging capabilities [59,60]. These agents, specially NO, have proven effect against virus, through a mechanism involving TLR7-myeloid differentiation primary response 88 (MyD88). Alternative eosinophils' responses against different viruses comprises further activation and secretion of a massive array of molecules such as IFN regulatory factor (IRF)-7, NOS-2, IFNβ,ribonucleases like EAR-1 and EAR-2, or interleukins and chemokines like IL-6, IP10, CCL2, and CCL3; all of them with variable effects in viral clearance [26,51,61,62] .

One of the most curious lines of antiviral defence that eosinophils can achieve is mediated through extracellular traps (ET). Yousefi and co-workers showed that eosinophils are able to catapult without dying their mitochondrial DNA, which forms extracellular structures able of trapping and killing bacteria [63]. A recent study performed by Silveira et al showed that this

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mechanism is also active in eosinophils from asthmatic mice exposed to RSV, increasing the quantity of DNA detected in bronchoalveolar lavage fluid (BALF) [64]. These results show that eosinophils also release extracellular DNA traps that have a prominent role in viral defence, similar to the traps released by neutrophils [65].

Finally, but no less important, eosinophils are able to release typical antiviral type 1 cytokines like IFNγ, IL-2 and IL-12 stored inside their granules in response to stimulus like TNFα, IFNγ or IL-10 [66]. These molecules have proven effects in the clearance of viral infections, and thus show an alternative pathway by which eosinophils fight against viruses [67,68].

All these mechanisms are summarized in Figure 1.

2.2. The interaction between respiratory viruses and eosinophils

Eosinophils produce different molecules and present several mechanisms with potential antiviral activity as we have previously commented. In this sense, the role of eosinophils in a respiratory virus infection scenario is very relevant due to its connection with lung diseases with an important eosinophilic inflammatory component, such as asthma, in which respiratory viruses like RSV or human rhinovirus (HRV) are a key element in the trigger of asthma exacerbations. In this sense, RSV is the main virus isolated in children less than 3 years during winter season, whereas during the rest of the year HRV is the most common [69].

2.2.1. Respiratory syncytial virus (RSV) and vaccines studies with inactivated virus

RSV is an enveloped, negative-sense, ssRNA that belongs to *Paramyxoviridae* family. It is the most frequent virus causing bronchiolitis worldwide, followed by HRV [70]. After a primary infection by RSV, around 30 to 70% of infants develop bronchiolitis, being hospitalised 1% to

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3% of them and, being actually an important cause of pneumonia in adults, especially in elderly patients [71–73].

Recent studies have linked several aspects and parameters of RSV infection with eosinophils and eosinophil degranulation products that bind to the dual capacity of eosinophils to develop a type 2 immune response and their ability to produce type 1 cytokines with pro- and anti-inflammatory properties [18,66,74,75].

In the case of RSV, both in infected eosinophils, these cells exposed to infected epithelial cells, or eosinophils activated through TLR7 ligands, this virus triggers several mechanisms such as production of IL-6, ECP and EDN which have antiviral activity due to their RNase capacity or the overexpression of CD11b, an activation marker in eosinophils [30,55,62,76]. Moreover, production of NO by iNOS due to TLR-7 has been postulated as one of the most important elements in antiviral mechanisms by which eosinophils decrease the viral titre, probably depending these antiviral effects on MyD88 adaptor protein-dependent signalling [26,59,77]. Besides, an important eosinophil recruitment to the RSV infection area is mediated through RANTES, which has been detected in the supernatant of bronchial epithelial cells infected by this virus [78] (Figure 2, upper left panel).

The first common scenario documenting the connection between RSV and eosinophils was at the end of the 60s during a trial of a formalin-inactivated RSV (FI-RSV) vaccine [79]. In this study, 80% of immunized infants were hospitalised compared with 5% hospitalization without deaths in the control group. Authors concluded that neutralizing and protective antibodies were not produced; furthermore, vaccinated children developed a hypersensitivity response to the virus antigens with severe pneumonia and bronchoconstriction. Histological analysis of lung biopsies

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from two children that finally died due in this trial, showed a relevant tissue eosinophilia and the deposition of antibody-virus complexes. One or more of these characteristics have been replicated with FI-RSV in other species [80,81]. In a murine model of immunization followed by an intranasal virus challenge with FI pneumonia virus of mice (PVM), a rodent pneumovirus pathogen related to RSV; showed similar results comprising pulmonary hypereosinophilia without serum-neutralizing antibody response [82]. Similar to this fatal trial in infants, another clinical study obtained negative results too. In this study performed by Kapikian and collaborators, 69% of immunized infants developed pneumonia in contrast to 9% observed in children of unimmunized control group [83]. Ulterior studies have proposed that these granulocytes inhibit in part RSV and the equivalent in mice (PVM) via their granule RNases, ECP, and EDN, degrading viral RNA genomes [55,56,58].

Multiple animal models have been developed attempting to elucidate the mechanisms involved in the called “vaccine-enhanced disease” (VED) or “immunopotentiation” described in the previous commented trials, just as the antiviral response and the best approach to achieve a safe and effective vaccine. Many mouse models of FI-RSV VED have confirmed that a hallmark of this disease is the pulmonary eosinophilia linked to a pronounced production of type 2 cytokines, mostly IL-4, IL-5, and IL-13; playing IL-4 and IL-5 a relevant role in the observed immunopotentiation since interfering with their activity markedly diminishes the severity of disease [84–87]. So, contradictory results have been found related to the role of eosinophils in these murine models of RSV VED, although a negative role of this kind of granulocytes is the predominant tendency.

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Research group leading by Sang-Moo Kang has profoundly analysed the VED linked to RSV in several murine models publishing numerous manuscripts [88–91]. They developed different RSV models based in different vaccine formulations or infection/immunization type [88–91]. Thus, they analysed and compared the immune response unleashed by FI-RSV vaccine or immunization performed through virus-like nanoparticles carrying RSV fusion proteins (F-VLP), F protein in a soluble form or an RSV combination vaccine composed by F-encoding plasmid DNA and virus-like particles containing RSV fusion (F) and attachment (G) glycoproteins (FFG-VLP), being safer and with a higher protective efficacy the models in which the viral proteins have been encapsulated compared to those employing the soluble protein F or FI-RSV vaccine [88,89,91]. These safe formulations preferentially elicited IgG2a antibody and type 1 immune responses. All of them showed the presence of eosinophils as important element of an inflammatory status and linked to a worse evolution of disease, characterizing the RSV VED. In this sense, others authors have considered that RSV-specific $CD8^+$ T memory cells are the crucial cellular type to avoid RSV VED, which is characterized by weight loss, bronchial hyperresponsiveness and pulmonary eosinophilia, considering that eosinophils play a harmful role [92,93]. Furthermore, through a murine model, Pennings and collaborators postulated that blood mRNA analysis could be used to identify an unfavourable type 2 response in VED situation [94]. So, they observed an increase of *Ear 1/2/3/6* expression in blood transcriptome during VRS-VED in mice that previously had received FI-RSV vaccine, being these genes associated with eosinophils [94].

In contrast, some authors considered that eosinophils could play a dual role or, even, protective functions in RSV infection, avoiding the VED [78,95]. Su et al studied the role of IL-5, eotaxin

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and eosinophils in a model of vaccine-enhanced RSV disease. In the IL-5- and eotaxin-double-deficient mice, accumulation of eosinophils in the lungs was reduced in conjunction with an augment of the virus titre. The transfer of eosinophils to both deficient mice models was accompanied by a rapid clearance of RSV through antiviral mediators produced by eosinophils as nitric oxide [78]. So, in a type 2-polarized inflammatory response, the migration and subsequent eosinophil activation in the lung has both inflammatory and antiviral functions. In the previous year to this document, Percopo and collaborators showed that eosinophils promote survival in the context of a lethal infection by PVM, although they present pathophysiological features in a type 2-polarized environment [58]. Before to these manuscripts, a model with eosinophil-deficient mice previously immunized with a vaccine comprised by RSV attachment glycoprotein produced the development of increased weight loss and clinical illness [95].

Even some manuscript suggest that eosinophils are not necessary the critical immune component associated with immunopotentiation linked to administration of FI-RSV vaccine [96,97]. In both cases, it is remarkable the absence of eosinophils in the inflammatory cellular infiltrate. All disease parameters associated with FI-RSV VED were mediated by $CD4^+$ T cells, including airway obstruction, weight loss, and airway hyperresponse. The depletion of $CD4^+$ T cells led to a significant amelioration of all disease parameters [96].

All these contradictory and significant different results conduce to theory that eosinophils might induce an antiviral response against respiratory viruses depending of specific situations causing, simultaneously, an excessive immune response trying to eliminate the virus and leading to host damage, being a double-edged sword [98]. A more profound knowledge of role and mechanisms of eosinophils in different context is necessary. In this sense, Flores-Torres *et al* suggest that comparing the response from lung-resident and traditional eosinophils against respiratory viruses could be interesting [99].

2.2.2. Other respiratory viruses: human rhinovirus (HRV), influenza and parainfluenza

Human Rhinovirus (HRV)

HRVs, contrary to RSV, are positive-sense, ssRNA virus member of the *Picornaviridae* family. As we have previously mentioned, HRV is the most frequent virus identified in the upper respiratory tract infection and it is closely linked to asthma exacerbations, mainly in childhood asthma development just like to chronic pulmonary disease exacerbations, severe bronchiolitis in infants as well as lethal pneumonia in elderly and immunocompromised adults (mainly rhinovirus C) [100,101]. So, this virus takes on relevance in asthma scenario in which eosinophils are one of the main actors.

The anti-HRV activity by eosinophils could be mediated by binding of these granulocytes to HRV-16 through ICAM-1, acting as an APC inducing the $CD4^{+}$ T cell proliferation and IFN-γ production which increases the expression of TLR-7 on eosinophils, suggesting the eosinophil-T cell cooperation [45] (Figure 2, lower right panel).

Intriguingly, in asthmatic patients, their eosinophils displayed a reduced capacity to bind virus, and HRV induces a loss of asthma control [102].

All this information takes importance in the era of new biological asthma treatments focused on eosinophils and their cytokines elimination. In these patients, a diminished CD69 expression on eosinophils surface was observed in HRV-16 infection and it is strongly correlated with loss of asthma control previously mentioned [102]. Moreover, the depletion of eosinophils as consequence of Mepolizumab treatment, a humanized monoclonal antibody that passively

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eliminates eosinophils through IL-5 removing, followed by a HRV-16 challenge resulted in enhanced viral titre, proving the relevance of eosinophils in viral respiratory infections [103]. In biological treatment, the extinction of eosinophils generates an important change of innate immune response during viral infections, increasing the importance of macrophages function.

Influenza virus

Influenza virus belongs to *Orthomyxoviridae* family, is an enveloped, negative-sense, segmented ssRNA virus [104]. Influenza A virus (IAV) is able to cause a wide range of severity, from mild to lethal infection, inducing high morbidity and mortality, in fact, it is the origin of seasonal epidemics and global pandemics, being IAV infection a major cause of public health concern [105,106]. Eosinophils are not kept in mind as the main effector cells playing in the first line antiviral immune response. However, as previously mentioned, during the 2009 H1N1 pandemic, epidemiological data suggested that asthmatics, probably due to their pulmonary eosinophilia, have a less likelihood of suffer from IAV morbidity and mortality [107,108]. In this context, a combined model of acute allergy and influenza infection shows higher eosinophil number in the airways and a rapid viral clearance; thus, it suggests that eosinophils are conferring protection from IAV-induced airway damage compared with infected mice with chronic asthma, supporting the hypothesis and results of a previous study [109,110]. Therefore, eosinophils could be important mediators in anti-influenza immunity in special populations of patients with a type 2-polarization of immune response.

Eosinophils develop multifaceted functions during an IAV infection through piecemeal degranulation, producing several cytokines and mediators like NO as well as these cells have the capacity to prime $CD8^+$ T cells acting as APC [44,61]. Moreover, an abortive infection has also

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suggested in eosinophils infected by influenza virus, being a passive mechanism through these leukocytes limit the viral expansion [44]

Also, in a paediatric population with acute pneumonia due to influenza virus a rise in serum IL-5 levels and peripheral eosinophilia were observed suggesting that eosinophil recruitment may be necessary in the late-stage for host defence against influenza virus [111]. Pulmonary eosinophilia has been observed in IAV-infected mice, proposing that this eosinophil recruitment could be mediated by IL-5 or CCL-5, cytokines produced during IAV infection [112,113] (Figure 2, l lower left panel).

Human Parainfluenza virus (hPIV)

HPIV, is an enveloped, negative-sense, non-segmented ssRNA member of *Paramyxoviridae* family. Parainfluenza is one of the several viruses causing asthma exacerbations, detected in up to 18% of adult airways during these acute episodes [114].

In the same way that viruses previously mentioned, eosinophils seem to carry out an antiviral function through TLR-MyD88 pathway [26]. In an asthmatic scenario, eosinophils could play an antiviral important role during an infection by PIV, reducing the viral content of lungs, effect that is reverted when IL-5 is blocked, which suggests that the prior effect is originated by eosinophils recruitment to the infected area (Figure 2, upper right panel) [115].

Drake and colleagues studied the effects of eosinophils on parainfluenza virus, both *in vivo* by means of an infection in mouse airways and *in vitro* in isolated human eosinophils [116]. The authors propose a dual functionality via proactive and passive mechanisms. These granulocytes generate NO that inhibits parainfluenza activity; however, eosinophilic RNases do not seem to be involved in antiviral effects. The passive mechanisms are characterized by an abortive infection

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meaning parainfluenza is able to infect eosinophils but the propagation of infectious viral progeny fails, intercepting the viral expansion [116].

The phenomenon observed in FI-RSV vaccines has also been detected in formalin-inactivated version of parainfluenza virus, observing a marked peribronchiolitis, perivasculitis, and an alveolar cellular infiltration [117].

Thus, eosinophils have beneficial effects during viral infections; however, and perhaps likely, the eosinophil ability to respond to viruses promotes an excessive and ultimate detrimental airway inflammatory response in subjects with asthma leading to global negative perception about the role of eosinophils in respiratory diseases.

3. THE INTERACTION OF EOSINOPHILS WITH CORONAVIRUS

3.1. Eosinophils and their role against SARS-CoV-1, and in the immune responses in severe acute respiratory syndrome coronavirus (SARS-CoV) vaccines

Since the epidemic caused by the previous SARS-CoV late in 2002 in China, several approaches have been carried out focused on the development of a vaccine that could protect against this human coronavirus and other potential zoonotic coronaviruses. Several vaccine candidates were developed against the first SARS-CoV based on virus-like particles, whole inactivated virus, recombinant vaccines or plasmid DNA vaccines, among others [118,119]. However, one of the most important factors to take into account for the development of an effective and safe vaccine that may protect against the agent causing COVID-19, SARS-CoV-2, and other potential coronaviruses, is to avoid undesired immunopathological effects as occurred with the respiratory syncytial virus vaccine already mentioned [120,121]. Indeed, a similar pathology was observed

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during the development of vaccines for the previous SARS-CoV, showing in most cases an exacerbated immune response characterized by a pathologic infiltration of eosinophils in the lungs after challenging previously immunized animal models [119].

One of the first works in the development of coronavirus vaccines was performed by Deming and co-workers in 2006. They employed Venezuelan equine encephalitis virus replicon particles (VRP) expressing the spike (S) protein or the nucleocapsid (N) protein. The first one showed neutralizing antibodies production conferring short-protection in young mice but almost no effect in senescence animals; however, the VRP-N vaccine, not only was unable to induce protection but also caused immunopathology with high eosinophil infiltration in the lungs of challenged mice [122]. Some years later, in 2011, the same group studied the effect of a whole virus double-inactivated vaccine. They observed the same immunopathologic effect in lungs with an exacerbated eosinophil infiltration, and they concluded that this effect is caused by the presence of the N protein in the vaccine [123].

Another recombinant viral particle vaccine was designed by Yasui and collaborators. They developed several vaccine candidates, either expressing all non-structural proteins together or expressing non-structural proteins separately. In the cases where N protein was present, not only a positive immune response with antibody production was not observed, moreover, an exacerbated immune response was reported with high eosinophil, neutrophil and lymphocyte infiltration in the lungs [124].

Other vaccine based in the receptor-binding domain (RBD) of the SARS-Cov S protein was developed by Du and colleagues. They fused this peptide to the Fc of the human IgG. The RBD-Fc vaccine showed a potent ability to induce neutralizing antibodies after inoculation in BALB/c

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mice [125]. In addition, no pathological damage was observed in the lungs of the animals, and remaining, for at least 6 months, the neutralizing antibodies [125]. This approach seemed encouraging, however to induce an effective antibody response higher adjuvant concentration and more boosters are needed compared with other vaccines [118,119].

Some years later, in 2012, Tseng and co-workers evaluated four SARS-CoV vaccine candidates in regard to the effectiveness, safety and immunogenic potential. The vaccines were: 1) a whole virus vaccine double-inactivated with formalin and UV irradiation (DIV) developed by Spruth and colleagues; 2) a whole virus vaccine inactivated with β-propiolactone (BPV); 3) a recombinant S protein vaccine (SV) produced in insect cells and purified by chromatography; and 4) a virus like-particles (VLP) vaccine containing the SARS-CoV S protein and the N and M proteins from the mouse hepatitis coronavirus [126–129]. The conclusions of this comparative work were that the four vaccines studied induced a type 2 immunopathology in lungs characterized by high infiltration of eosinophils in animals challenged with the virus after vaccination. However, in addition to this undesired effect, the four vaccines also induced neutralizing antibodies that avoided a lethal disease compared to controls [130]. An important point highlighted in this paper was the fact that the use of alum as an adjuvant could bias the immune response to a type 2, although the same pathologic effect was observed using vaccines without alum [130].

Trying to elucidate whether the adjuvant might bias the immune response towards a type 1 or type 2 response, Honda-Okubo and collaborators compared a range of recombinant S protein or whole-virus vaccines in a murine model using different types of adjuvants, including alum, CpG and Advax, a new delta inulin-based polysaccharide adjuvant [131]. They proposed the use of

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inulin-based adjuvants rather than alum since no eosinophilic immunopathology was observed in the lungs and an enhanced T cell and humoral response may be achieved including this adjuvant in vaccine formulations [131].

Other alternative was proposed by Iwata-Yoshikawa and colleagues in 2014. They used a whole UV-inactivated vaccine for immunization of BALB/c mice and observed that the addition of a toll-like receptor (TLR) agonist such as polyinosinic:polycytidylic acid (poly-IC), polyuridylic Acid (poly-U) or lipopolysaccharide during vaccination induced a high level of neutralizing antibodies against SARS-CoV but a non-pathogenic eosinophil infiltration in the lungs, probably due to a balance between the type 2 and type 1 response mediated by the TLR stimulation and also lower levels of type 2 interleukins such as IL-4 and IL-13 in the lungs [132].

All that is already known will help researchers in the development of an effective and safe vaccine that could protect population against SARS-CoV-2 and hopefully to other potential coronaviruses affecting humans. Moreover, all that has been learnt during SARS-CoV vaccine development put emphasis on the importance of not to take shortcuts and to prioritize human safety.

3.2. Current knowledge and perspectives regarding eosinophils in COVID-19 disease (SARS-CoV-2)

COVID-19 is a new coronavirus disease that has declared a Public Health Emergency of International Concern on 30 January 2020 by WHO, becoming one of the worst infection disease outbreaks known, with over 6.9 million cases and 400,000 death so far and 2116 countries, areas or territories with cases.

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Early observations in the COVID-19 patients described eosinopenia or low blood eosinophil count in hospitalized COVID-19 patients and more importantly, seems that it correlated with the severity of the disease or with a poor prognosis [133–138]. Accordingly, samples from lung biopsies or BAL from COVID-19 patients show an aberrant and massive macrophages inflammation, but eosinophils were not found, and the inflammatory profile observed in lungs from COVID-19 patients is basically Th1, Th17 phenotype [139–142].

Eosinopenia is not an exclusive characteristic of COVID-19 disease. A decrease of eosinophils blood count has been observed in different acute inflammation situation as pneumonia, but not in chronic respiratory disease as asthma [143–148].

But this asseveration in COVID-19 patients is controversial. Most of the studied patient series are made in the same geographic region, as China, and the number of patients in studies is short. Lippi *et al* reviewed the literature about eosinophils and COVID-19 and they suggested that eosinopenia may not be associated with unfavourable progression of COVID-19; this conclusion is based in data from 294 patients [149].

By contrast, Sun et al declare that eosinophils count was significantly decreased in patients with severe disease, so eosinopenia was a feature of higher levels of severity, but the limitation of the study is similar to as above, the short number of studied patients (*n*=63) [150].

An important fact is that eosinophil levels improved in patients prior discharge, suggesting that eosinophil resolution may be an indicator of improvement of clinical condition [151]. Certain authors speculate that the aspects of type 2 immune response, including type 2 cytokines (IL-4, IL-13, etc.) and accumulation of eosinophils, might provide potential protective effects against COVID-19 [152].

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The immune mechanism of eosinopenia in COVID-19 remains unclear, but is likely multifactorial, involving inhibition of main steps of eosinophil life cycle (ontogeny, rolling, adhesion and migration), apoptosis induced by type 1 IFN during the acute infection or relation to eosinophil consumption by eosinophil antiviral actions [153,154]. Thus, Jesenak and collaborators, considered that eosinopenia could be either the sign or the symptom of host exhaustion due to clearance of COVID-19 virus [155].

In conclusion, eosinopenia in COVID-19 seems a frequent feature but studies with a larger number of patients and on the eosinopenia mechanism should be carried out to confirm and clarify the role of eosinophils in this emerging disease.

Conflict of interest

VdP has received honoraria (advisory board, speaker) and/or institutional grant/research support from Astra-Zeneca and GSK, thee other authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. The eosinophilic response against viral infection comprises the following mechanisms: recognition of viral molecules (RNA) by the Toll Like Receptors (TLRs) 3, 7 and 9, through Myeloid differentiation primary response 88 (MyD88) and the prolyl isomerase Pin1 eliciting the expression of Interleukin (IL)-6 and granule proteins; antigen presentation to T cells trough Mayor Histocompatibility Complex (MHC)-II and molecular co-stimulation by CD80/86 and CD40L causing T cell activation and secretion of eosinophil chemoattractants as IL-4 and IL-5; and finally secretion of antiviral molecules including eosinophil granule proteins like eosinophil derived neurotoxin (EDN), Eosinophil Cationic Protein (ECP) or Mayor Basic Protein (MBP) and Reactive Oxygen Species (ROS) and Nitric Oxide (NO), alongside interleukins with both antiviral and immune enhancing properties like IL-2, IL-8, IL-12 and Interferon (IFN)γ/β. Eosinophils are also able to secrete mitochondrial DNA traps, that are Eosinophil Extracellular Traps (EETs) with the ability to catch and destroy virus.

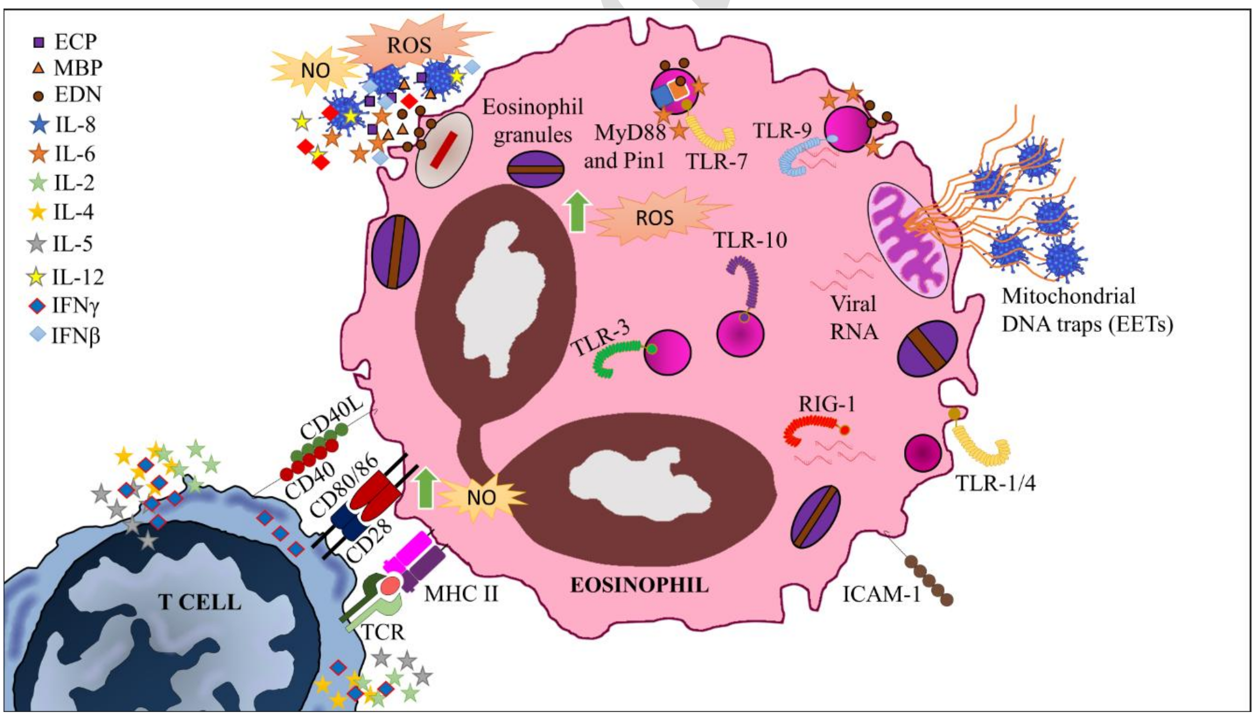

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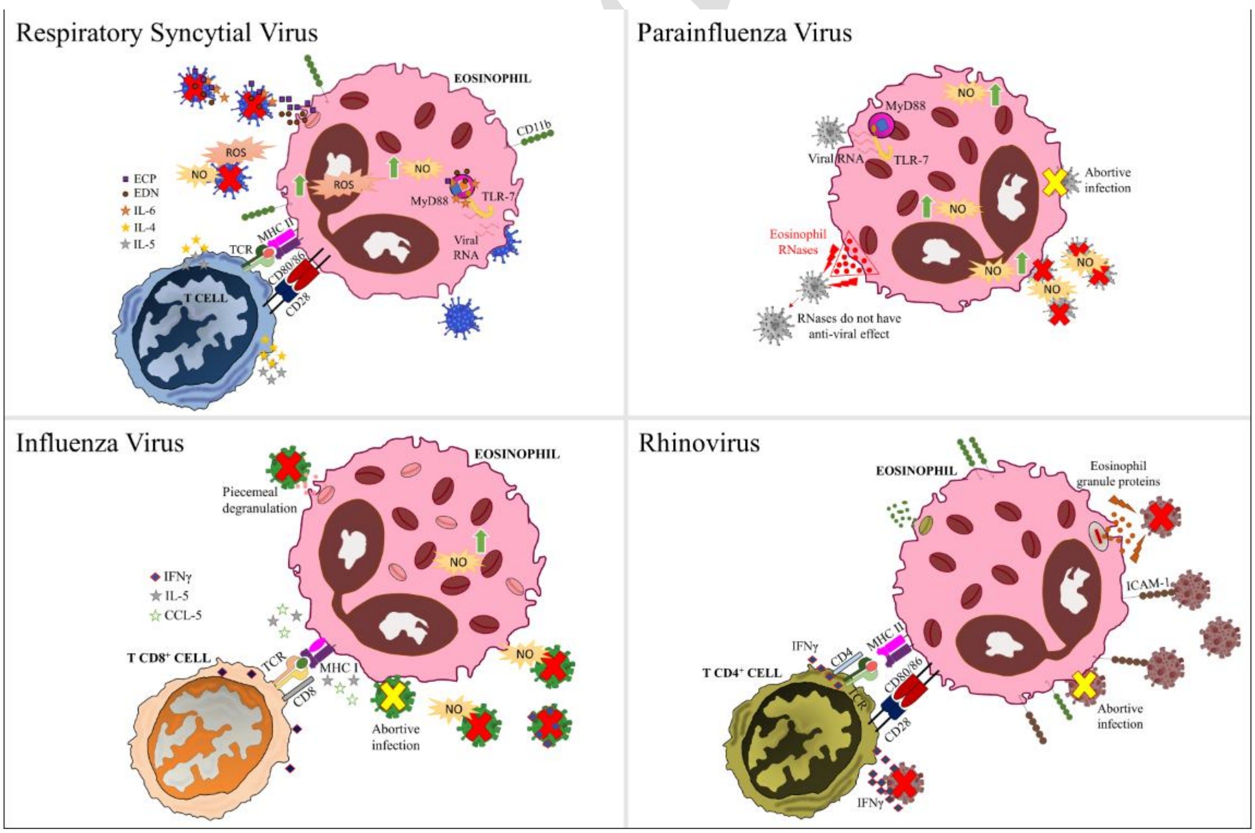
Figure 2. The specific response of eosinophils against respiratory viruses. Respiratory syncytial virus (RSV): Eosinophils infected by these viruses are able to sense viral RNA particles by Toll like receptor (TLR)-7/Myeloid differentiation primary response 88 (MyD88) and secrete nitric oxide (NO), interleukin (IL)-6, reactive oxygen species (ROS) and eosinophil-derived neurotoxin (EDN), and upregulate CD11b. They also interact with T cells and present viral antigens by Mayor Histocompatibility Complex (MHC)-II binding with T Cell Receptor (TCR) and co-stimulation by CD80/86 ligation to CD28, causing secretion of pro-eosinophil IL-4 and IL-5. Parainfluenza virus: Similarly to RSV, eosinophils' TLR7 is activated by viral RNA inducing NO secretion, while eosinophil peroxidase (EPO) does not seem to be needed for viral clearance. Abortive infection is another mechanism by which viral replication is inhibited by these granulocytes. Rhinovirus: The eosinophil receptor Intercellular Adhesion Molecule 1 (ICAM-1) is able to bind this virus, while antigen presentation through MHC II and CD80/86 to CD4+ T cells induces the secretion of antiviral interferon IFNγ by these cells. Influenza virus: The main response of eosinophils attracted to the infectious focus by IL-5 and CCL-5 consist in the secretion of NO, piecemeal degranulation and abortion of the viral replication. Antigen presenting by MHC I to CD8+ cytotoxic T cells also induces these T cytotoxic cells to secrete antiviral IFNγ.



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