Eosinophils and their Interactions with Respiratory Virus Pathogens

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Abstract

Eosinophils are implicated in the pathophysiology of respiratory virus infection, most typically in negative roles, such as promoting wheezing and bronchoconstriction in conjunction with virus-induced exacerbations of reactive airways disease and in association with aberrant hypersensitivity responses to antiviral vaccines. However, experiments carried out *in vitro* and *in vivo* suggest positive roles for eosinophils, as they have been shown to reduce virus infectivity in tissue culture and promote clearance of the human pathogen, respiratory syncytial virus (RSV) in a mouse challenge model. The related natural rodent pathogen, pneumonia virus of mice (PVM) is highly virulent in mice, and is not readily cleared by eosinophils *in vivo*. Interestingly, PVM replicates in eosinophils and promotes cytokine release. The molecular basis of virus infection in eosinophils and its relationship to disease outcome is currently under study.

Keywords

Eosinophils; Inflammation; Virus; Ribonuclease; Cytokine

Enigmatic Eosinophils

Eosinophils are among the most enigmatic of all cells of the immune system. In the recent past, much about their basic biology, the nature of their effector functions, and roles played in various disease states has come under renewed scrutiny [1,2]. For example, the tacit assumption that eosinophils provide protection against infection with helminthic parasites is now a subject of profound disagreement [reviewed in 3]. Likewise, eosinophils have long been perceived as major pathophysiologic mediators in respiratory allergy and asthma, yet therapeutic trials with humanized anti-interleukin-5 monoclonal antibodies [4,5] and studies with eosinophil-ablated mouse models [6] have created substantial controversy. Finally, even the most treasured belief, that eosinophils mediate all significant effector functions via degranulation of cationic proteins has been called into question [7]. At the same time, the role of eosinophils in respiratory virus infection, a connection that had more or less gone unnoticed, has emerged as one of the many new and exciting areas of study linking infectious pathogens and allergic responses [8–11].

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Eosinophils, Asthma and Antiviral Host Defense – a Double-Edged Sword?

The double-edged sword is a concept that reflects the competing beneficial and detrimental features of a given physiologic/pathophysiologic response, and has been used to formulate our current understanding of the physiology of the neutrophil and diseases involving dysregulation of neutrophil recruitment and activation [12]. For example, although neutrophils are clearly effective at promoting host defense against bacterial pathogens via degranulation, phagocytosis, and production of reactive oxygen species, the dysregulation of these essential, beneficial responses can leadto reperfusion injury and adult respiratory distress syndrome (reviewed in 13).

We would like to present the possibility of a clear and definitive parallel when considering eosinophils and the pathogenesis of reactive airways disease. There are now several studies demonstrating a striking correlation between severe respiratory syncytial virus (RSV) infection in infancy and the development of reactive airways disease in later childhood years (recently reviewed in 14,15). Respiratory virus infections, including RSV, are a universal affliction of infancy, childhood and beyond. Once reactive airways disease is established, subsequent respiratory virus infection is clearly accepted as among the most common causes of disease exacerbation [14,15]. We propose the possibility that asthma and reactive airways disease represent a dysregulation of what are otherwise essential and beneficial responses. Whatever causes this dysregulation to be established -whether it is infection with a specific respiratory virus at a specific developmental stage in a genetically and/or environmentally susceptible individual or some subset of the aforementioned - it is intriguing to consider the possibility that the responses characteristic of the asthmatic state represent the dysregulation of responses primarily directed toward a beneficial role in promoting innate antiviral host defense.

Eosinophils, Evolution and Structure

While all mammals have eosinophils, easily recognized on the basis of nuclear morphology and cytoplasmic granule staining [Figure 1], there are striking species-specific differences. For example, human eosinophils express the high affinity IgE receptor, FcεRI, while mouse eosinophils do not. Furthermore, the Charcot-Leyden crystal (CLC) protein, a major component of human eosinophils, has no functional correlate in mouse eosinophils, nor any identifiable ortholog in the mouse genome. Mouse eosinophils express Siglec F, a sialic acid-binding Ig-like lectin that is a functional but highly divergent ortholog of the human eosinophil protein, Siglec 8 [18]. *Vis à vis* respiratory inflammation, mouse eosinophils have distinctly different responses to chemotactic cytokines than human eosinophils [19]. Equally remarkable, and the subject of much of our group's research, are the eosinophil secretory ribonucleases, EDN and ECP, which are present in high concentration in the eosinophil granules. EDN and ECP are among the most rapidly evolving functional coding sequences known among primates [20,21] so much so that, as shown in Figure 2A, orthologous sequences in non-primate mammals cannot be detected by hybridization methods; the mouse eosinophil-associated RNases, a large cluster of equally rapidly-evolving non-overlapping sequences that have emerged via rapid duplication and gene sorting, are distant orthologs of their human counterparts [22,23; Figure 2B]. Despite rapid evolution, all coding sequences identified have elements necessary to maintain the canonical RNase A tertiary structure and catalytic elements necessary to support enzymatic activity.

Initial Hypothesis

Although eosinophils are typically perceived as contributing to respiratory dysfunction, including tissue damage and bronchoconstriction and are associated with asthmatic disease or hypersensitivity responses, either alone or in association with respiratory virus infection, from an evolutionary standpoint, a cell type will not persist in nature if all or most of its major

functions are deleterious to the host. As such, we considered the possibility that there are "yin-yang" aspects to eosinophilic inflammation, much like the dual nature of neutrophilic inflammation in the setting of bacterial infection. Initially, we proposed that eosinophils, relying all or in part on their array of powerful and rapidly evolving secretory ribonucleases, could play a role in promoting host defense by direct targeting of ribonucleolytically-vulnerable single-stranded RNA genome of respiratory virus pathogens such as RSV.

Eosinophils Reduce Virus Infectivity *In Vitro*

To begin, we asked very simple questions. What was the impact of adding isolated human eosinophils to respiratory syncytial virus virions prior to infecting target cells in culture? What we found was fairly straightforward [Figure 3A]. When eosinophils were added in increasing concentration to a fixed number of viruses, a dose-dependent inhibition of virus infectivity was observed [24]. This inhibition could be reversed if ribonuclease inhibitor was added prior to addition of the eosinophils, suggesting that the secretory ribonucleases were promoting this effect, and were necessary, if not wholly sufficient. We were also able to demonstrate a reduction in infectivity with recombinant EDN alone, and that this effect was dependent on EDN's ribonucleolytic activity. We are continuing to explore the mechanism of the antiviral activity, which is far from straightforward.

Eosinophils and Antiviral Host Defense *In vivo*

The first study to address this question was that of Adamko and colleagues [25] who were performing a study focused on M2 muscarinic receptor function and allergic responses. However, as part of this work, they explored infection with Sendai virus (family *Paramyxoviridae*, genus *Respirovirus*) in guinea pigs initially sensitized with ovalbumin. The authors found that reducing the number of eosinophils in bronchoalveolar lavage fluid by systemic administration of anti-IL-5 antibody resulted in a pronounced increase in virus titer in lung tissue, providing the first evidence *in vivo* for a role for eosinophils in promoting antiviral host defense.

A second study, by Phipps and colleagues [26], addressed the question directly, exploring clearance of the human pathogen respiratory syncytial virus (RSV; family *Paramyxoviridae*, genus *Pneumovirus*) in wild type and eosinophil enriched IL-5 transgenic mice [Figure 3B]. As shown, RSV clearance proceeded more efficiently in the IL-5 transgenic mice and, even more interesting, the authors demonstrated that the full antiviral effects were directly dependent on intact signaling via MyD88, the adaptor protein utilized by TLRs, including TLR 7 to promote signal transduction. Thus, not only is this another clear demonstration of antiviral effects of mouse eosinophils *in vivo*, but the first suggestion of a role for TLRs – specifically TLR7, in promoting eosinophil-mediated antiviral activity.

Interestingly, initial studies suggest that eosinophils may not be as effective at combating infection with pneumonia virus of mice (PVM), a natural rodent pathogen that undergoes rapid and robust replication in mouse lung tissue. This will be discussed below after this pathogen model has been introduced.

Pneumonia Virus of Mice and Eosinophils

As we mentioned earlier, all mammals have eosinophils, but eosinophil structure and contents differ dramatically among species. Given these evolutionary considerations, we have focused much of our attention on developing a species-matched pathogen model. Pneumonia virus of mice (PVM; family *Paramyxoviridae*, genus Pneumovirus) is closely related to the human and bovine respiratory syncytial viruses [Figure 4; reviewed in 27–29]. Robust replication of PVM takes place in bronchial epithelial cells in response to a minimal intranasal inoculum. Virus

replication *in situ* results in local production of proinflammatory cytokines (MIP-1α, MIP-2, MCP-1 and IFNγ) and profound granulocyte recruitment to the lung. If left untreated, PVM infection and the ensuing inflammatory response ultimately lead to pulmonary edema, respiratory compromise and death, and as such, the infection resembles the more severe forms of human respiratory syncytial virus infection as experienced by human infants. Using this model, we have presented several combined antiviral/immunomodulatory strategies that have successfully reduced morbidity and mortality when administered to infected, symptomatic mice, and thus hold promise as realistic therapeutic strategies for virus infections in human subjects [30–32].

We have recently determined a means to study mice that have survived PVM infection. With a reduced inoculum size and volume, we observe virus clearance by day 9, while clinical symptoms and respiratory dysfunction persist through days 14 and 17 post-inoculation, respectively, even without subsequent provocation [Figure 5]. Via gene microarray and ELISA, we identified expression profiles of proinflammatory mediators that correlate with persistent respiratory dysfunction, including several eosinophil chemoattractants. This promises to be an extremely useful model for studying the role of eosinophils in post-infectious respiratory dysfunction *in vivo* [33]

Interestingly, initial studies suggest that eosinophils may not be as effective at clearing PVM from lungs of infected mice as they are against other virus pathogens. These results emerged from studies of mice vaccinated with formalin-fixed PVM antigens, designed to replicate the "enhanced disease" paradigm seen in response to formalin-fixed RSV vaccination (recently reviewed in 34). In wild type mice vaccinated with formalin-fixed PVM antigens, we observed profound pulmonary eosinophilia in response to challenge with active PVM infection. As anticipated, no eosinophilia is observed in response to PVM challenge of eosinophil-ablated ΔdblGATA mice vaccinated with the same formalin-fixed PVM antigens, yet lung virus titers from the PVM challenge are indistinguishable, suggesting that eosinophils are without impact (manuscript in review). Among the reasons for the differences among these models, PVM may have some means of disabling TLR7-mediated signaling, a feature underlying the efficient clearance of RSV. Similarly (and perhaps related to this?), PVM is more virulent *in vivo* than is RSV or even Sendai virus, and, as discussed below, PVM actually infects and replicates within mouse eosinophils. We are in the process of elucidating this finding and exploring the responses of infected vs. uninfected eosinophils.

Eosinophils are Targets of Virus Infection

As part of a separate initiative in our laboratory, we have recently described an *ex vivo* culture system which generates large numbers of eosinophils at high purity from unselected mouse bone marrow progenitors [Figure 6A, B]. In addition to expressing the appropriate mouse eosinophil granule proteins and cell surface antigens, we demonstrated that these cells produce characteristic cytokines and undergo chemotaxis toward mouse eotaxin-1 [35].

As part of our ongoing efforts toward understanding the cellular basis of PVM infection, we found that PVM replication was evident within these eosinophil cultures; clear evidence of virus replication emerges via quantitative RT-PCR detection of the virus SH gene as shown [Figure 6C]. Virus replication is accompanied by release of the cytokine, interleukin-6 [Figure 6D]. We have obtained similar results with mouse eosinophils isolated from IL-5 transgenic mice and we are examining eosinophils isolated from lung of mice undergoing PVM infection in vivo for evidence of active replication and cytokine release.

This finding elicits many critical questions. We are interested in determining precisely how the virus infects an eosinophil and in what subcellular compartment virus replication takes place. To answer this question, we are actively attempting to discern the ultrastructure of PVM-

infected mouse eosinophils. Furthermore, we would like to know whether or not replication disables the cell, diminishing, or perhaps augmenting its capacity to undergo chemotaxis, degranulation, and/or antigen presentation, and whether or not an infected eosinophil releases virions (ie… undergoes productive infection) and/or yields to apoptosis. Finally, it will be important to have a full accounting of the mediators released specifically by eosinophils in the lungs of PVM-infected mice in vivo, and to determine how and when these mediators contribute to the active infection and to the post-infectious recovery period.

Conclusions

We continue to explore the role of eosinophils and their interactions with respiratory viruses *in vivo* and *in vitro*. Human eosinophils reduce the infectivity of RSV in tissue culture and promote clearance of RSV from mouse lungs *in vivo*, the latter dependent on TLR7-MyD88 signaling. The rodent pathogen, PVM, is highly virulent in mice, and not readily cleared by eosinophils. We have determined that PVM replicates within eosinophils, and we are currently exploring the impact of this finding on the pathogenesis of the respiratory virus infection *in vivo*. We are intrigued by the possibility that replication within eosinophils may play a role in the pathophysiology of virus-induced asthma exacerbations.

Acknowledgments

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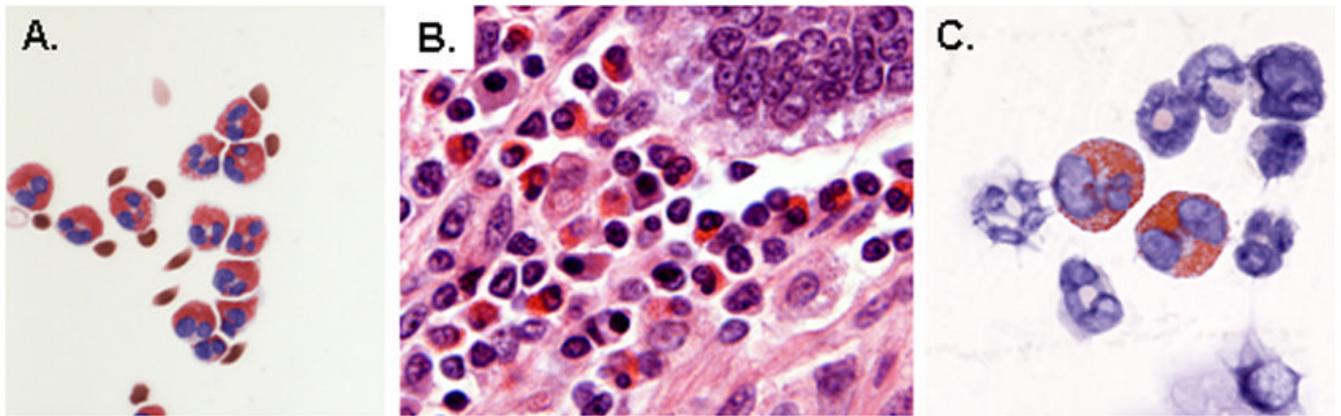
Figure 1. Eosinophils
(A) Human eosinophils isolated from peripheral blood by negative selection (B) Eosinophilic infiltrates in the intestinal wall of an Owl monkey, *A. trivirgatus* (image courtesy of Dr. Alfonso Gozalo, Comparative Medicine Branch, NIAID) (C) Eosinophils in mouse bone marrow cytospin. Eosinophils can be found in all mammalian species, and are easily recognized by their large, prominently stained cytoplasmic granules. Panels (A) and (C) reprinted with permission from reference [36].
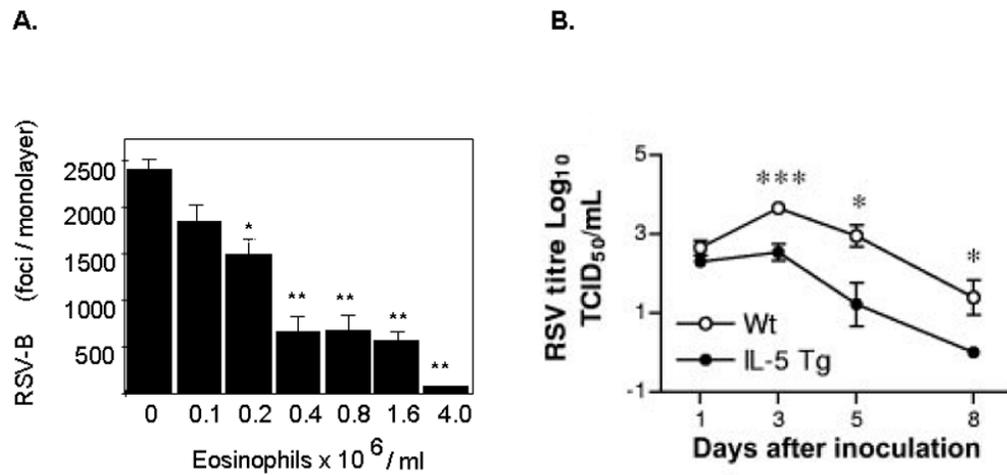
A.

B.

Figure 2. Evolution of eosinophil secretory ribonucleases
(A) Genomic DNA probed with the coding sequence of the eosinophil-derived neurotoxin
(EDN/RNase 2), demonstrating variant genomic structure among closely-related primates, and
no detectable hybridizing sequence among non-primate mammals. EDN and eosinophil
cationic protein (ECP) are among the most rapidly evolving functional coding sequences
identified among primate species. (B) Neighbor-joining tree documenting relationships among
mouse eosinophil-associated ribonucleases and human EDN and ECP. Panel (A) reprinted with
permission from reference [20].

Figure 3. Eosinophils and antiviral interactions with respiratory syncytial virus (RSV)
(A) Eosinophils reduce the infectivity of respiratory syncytial virus (RSV) in tissue culture.
Increasing concentrations of eosinophils function in a dose dependent fashion to reduce the
number of infectious virions as shown. (B) Eosinophils promote clearance of RSV in a mouse
challenge model. Virus is cleared more rapidly from the lungs of eosinophil-enriched
interleukin-5 (IL-5) transgenic mice when compared to wild type. Panels (A) and (B) reprinted
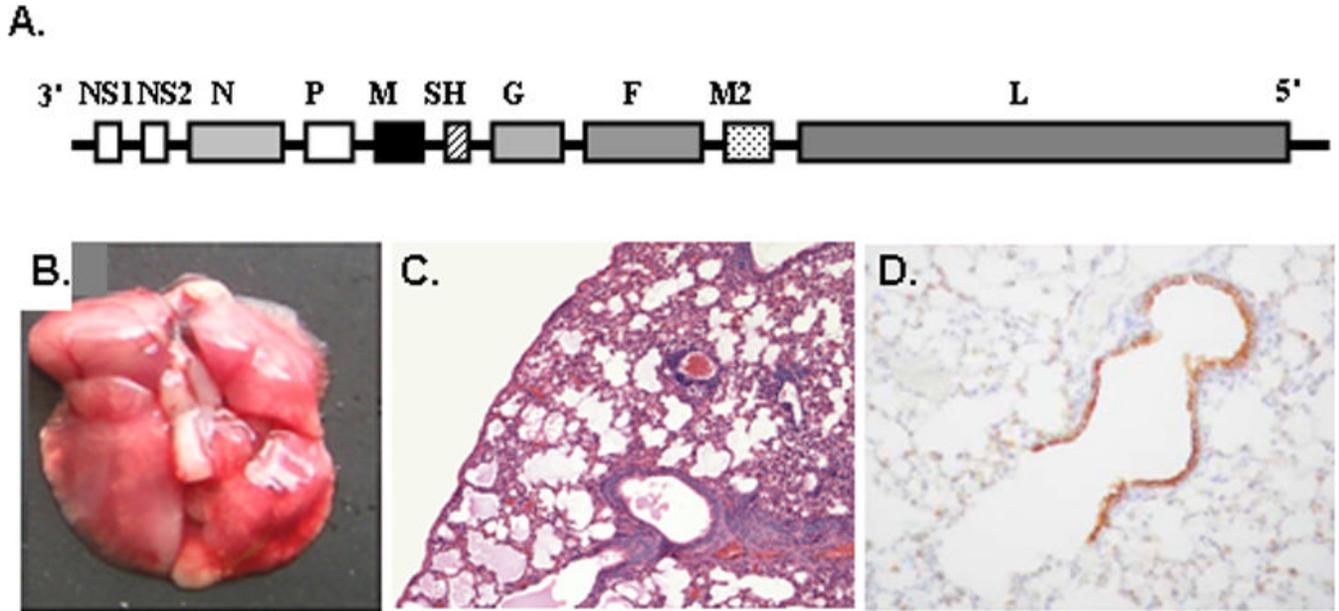with permission from references [24] and [26], respectively.

Figure 4. Pneumonia virus of mice (PVM) is a natural pathogen of rodent species
(A) PVM is a negative sense non-segmented single stranded RNA virus, family
Paramyxoviridae, genus *Pneumovirus*, gene order as shown. (B) Challenge with a minimal
inoculum (<100 pfu) results in a severe respiratory infection, with macroscopic evidence of
hemorrhage and (C) microscopic pathology including profound granulocytic inflammation and
edema. (D) Immunoreactive virus can be detected in bronchiolar epithelial cells. Panels (A),
(B), (C), and (D) reprinted with permission from references [29], [37], [38], and [33],
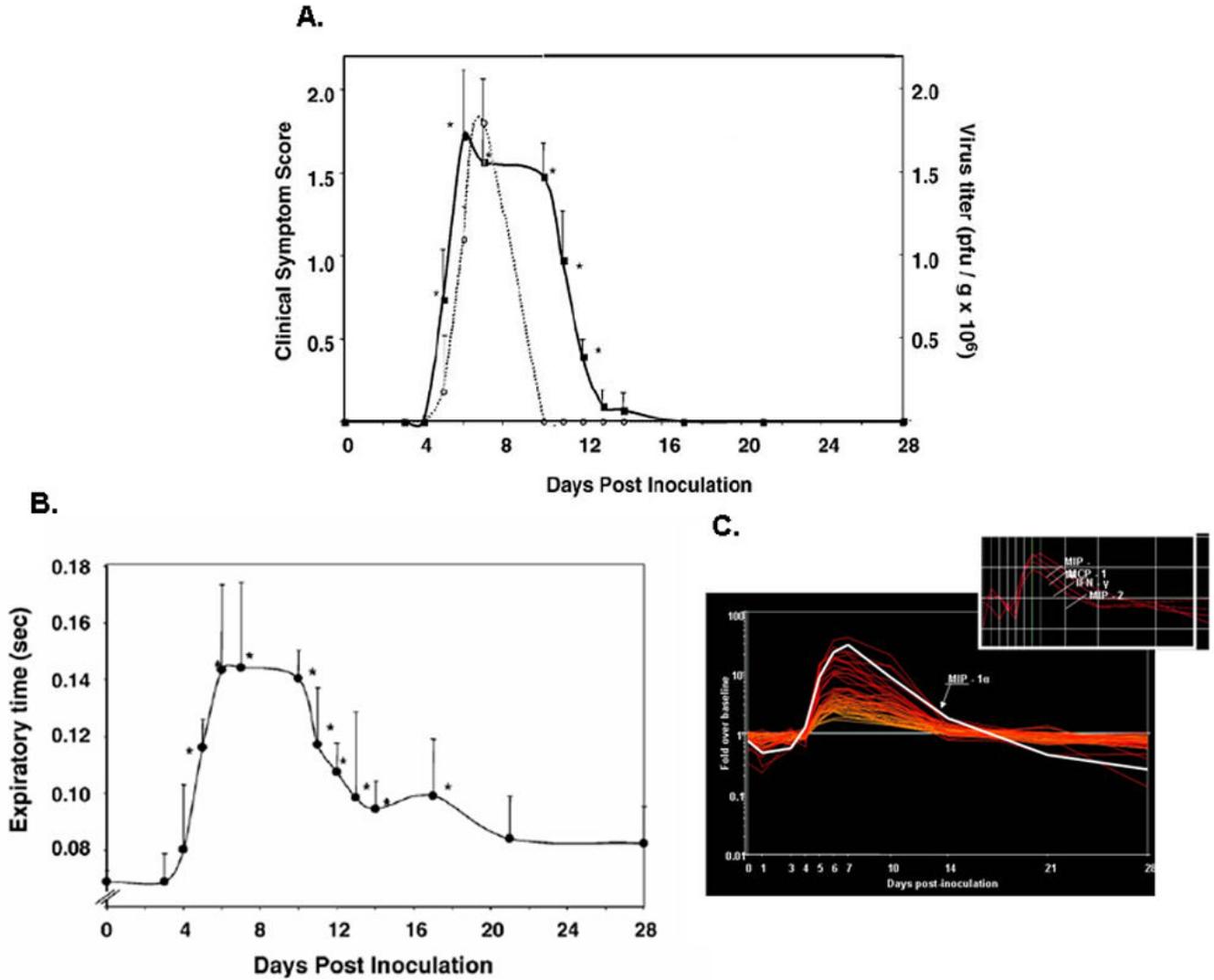respectively.

Figure 5. Virus replication and prolonged respiratory dysfunction
(A) As shown, virus replication (dotted line) peaks at day 7 in response to a minimal inoculum, and infectious virus can no longer be detected after day 10. In contrast, clinical symptom scores (full line; based on 6-point objective criteria) remain elevated through day 14, and (B) respiratory dysfunction, documented here as expiratory time, remains statistically above baseline through day 16. (C) Prolonged respiratory dysfunction correlates directly with persistent production of proinflammatory chemokines, despite resolution of the infection per se. Reprinted with permission from reference [33].
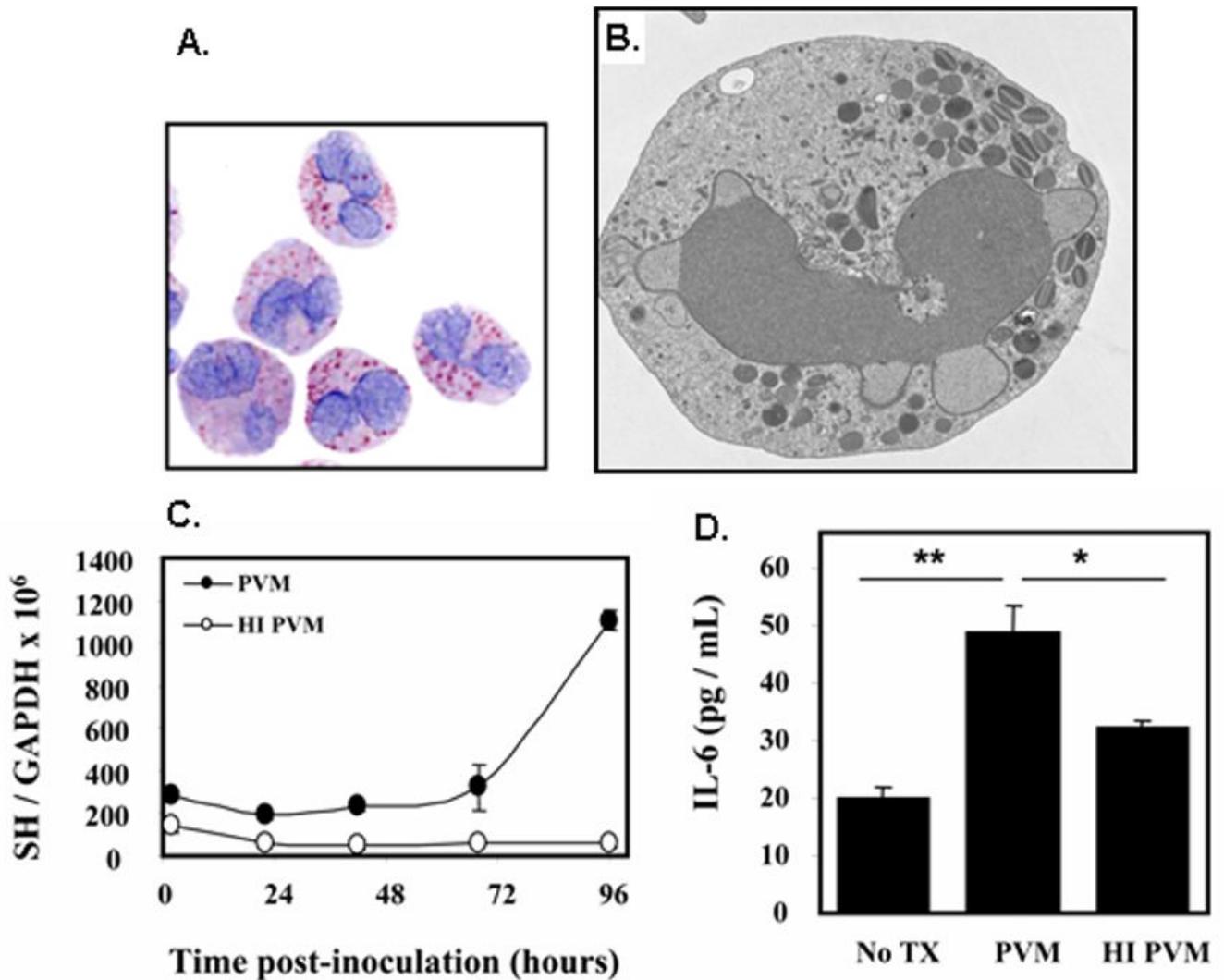
Figure 6. Eosinophils are targets of virus infection
(A) Mouse eosinophils grown in culture from unselected normal bone marrow, (B) Electron micrograph of cultured mouse eosinophil shown in (A) depicting normal nuclear, cytoplasmic and granule morphology; image courtesy of Dr. Elizabeth Fischer, Research Technologies Section, Rocky Mountain Laboratories, NIAID; (C) Replication of PVM in mouse eosinophils as determined by quantitative RT-PCR detection of the virus SH gene; no replication of heat-inactivated (HI) virus is observed. (D) Active replication is accompanied by release of interleukin-6. Panels (A), (C) and (D) are reprinted with permission from reference [35].