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Original Research

Quality over quantity; eosinophil activation status will deepen the insight into eosinophilic diseases

B. Hilvering [a,*], L. Koenderman [b]

[a] Dept. Pulmonary Medicine, Amsterdam University Medical Center, the Netherlands
[b] Dept. Respiratory Medicine and Center for Translational Immunology, University Medical Center Utrecht, the Netherlands

A B S T R A C T

Eosinophil associated diseases have gained much attention recently because of the introduction of specific eosinophil targeted therapies. These diseases range from acute parasitic infections to chronic inflammatory diseases such as eosinophilic asthma. In eosinophilic asthma an increased eosinophil cell count in peripheral blood is the gold standard for determination of the pheno-/endotype and severity of disease. Despite a broad consensus there is concern on validity of this simple measurement, because the eosinophil compartment is far from homogenous. Multiple tissues harbour non-activated cells under homeostatic conditions and other tissues, normally devoid of eosinophils, become infested with these cells under inflammatory conditions. It will, therefore, be clear that eosinophils become differentially (pre)-activated at different tissue sites in homeostatic and inflammatory conditions. This complexity should be investigated in detail as it is 1) far from clear what the long-term side effects are that are caused by application of eosinophil targeted therapies in a "one size fits all" concept and 2) real-world data of eosinophil targeted therapies in asthma shows a broad variety in the treatment response. This review will focus on complex mechanisms of eosinophil activation *in vivo* to create a better view on the dynamics of the eosinophil compartment in health and disease both to prevent collateral damage caused by aberrant activation of eosinophils ánd to improve effectiveness of eosinophil targeted treatments.

The eosinophil: an enigmatic cell type. Eosinophils are found throughout evolution and there is a clear co-evolution with the presence of helminths in the host [1]. Despite this general finding the actual function of eosinophils is yet to be established [2]. The long-lasting hypothesis of eosinophils as effector cells in defence against parasites has been disputed [3]. Ample studies with eosinophil knock-out mice showed no negative effect of absent eosinophils with respect to a parasitic host response. There are even examples of helminths that benefit from the presence of eosinophils, such as the *Trichinella spiralis*: an encysted larva in muscle tissue. Eosinophil derived cytokines, IL-4 and IL-10 diminish a host response during an infection with this helminth [4,5]. In addition, the first real-world studies of anti-IL-5 (Mepolizumab) in severe eosinophilic asthma actively looked into parasitic infections and did not find any infections in patients treated while living in parasite-endemic countries [6].

There is a current consensus that eosinophils have a variety of nuanced functions dependent on the tissue dwelling site. Eosinophils can be present in the gut, lung, uterus, mammary glands, adipose tissue and a variety of other sites [7,8]. Within these tissues they can be present in different states, ranging from a proliferative state in the bone marrow to a rolling state in the vasculature and an 'end-state' of EETosis (eosinophilic extracellular traps) in eosinophilic nasal polyposis and ear secretions of eosinophilic otitis media patients [9] (see Fig. 1). Eosinophil recruitment from the bloodstream requires activation of eosinophils resulting in 'rolling' on activated vascular epithelium and subsequent arrest [10]. After the arrest eosinophils can transmigrate through the vasculature, move through the extracellular matrix of the lung parenchyma and migrate through the epithelium into the airways to end up in their effector state [11]. Prior to the activation of eosinophils there is a state of pre-activation which is a sensitized or 'primed' state that is more sensitive for subsequent stimuli [12,13]. Some cells cannot be primed and are regarded inactive or 'refractory eosinophils' [14].

To study tissue eosinophils and measure their activation status has been proven difficult. It was hampered by technical limitations, such as the presence of RNA-se within the cell which made single-cell RNA-sequencing a real challenge. However, in the past few years innovations in single-cell analysis methods did lead to novel insights [15]. Specifically in-tissue analysis by confocal microscopy and flow-cytometry analysis on single cell suspensions of digested tissue brought more insights [16,17].

Despite the clear findings regarding the cell biology of eosinophils with respect to cellular functions in vitro and their underlying mechanisms, surprisingly little is known regarding these functions *in vivo*; particularly the role and the extent of activation of eosinophils in health

* Corresponding author.
E-mail address: b.hilvering@amsterdamumc.nl (B. Hilvering).

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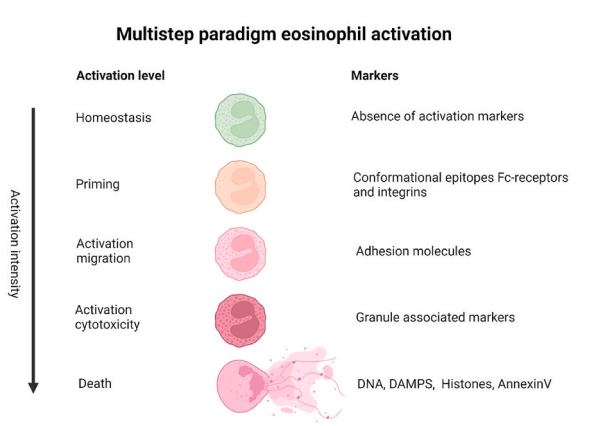

Fig. 1. Multistep paradigm of eosinophil activation: Increase in the extend of eosinophil activation shows a stepwise pattern. Each step is dependent on extrinsic activators and is characterised by different functional capacities. Created with biorender.com

and disease is yet to be elucidated.

The eosinophil compartment in the lung and its activation in homeostasis. In homeostasis the number of eosinophils in peripheral blood is typically very low, but it is influenced by known mechanisms such as allergies and diurnal variation and largely unknown mechanisms such as found in eosinophilic asthma. A large population study in Austria [18] has demonstrated that normal eosinophil levels in healthy individuals are around 100 cells/μL. Traditionally, eosinophil numbers up to 500 cells/μL were considered normal, but this has been refuted by many clinical studies targeting eosinophils in allergic diseases showing that eosinophil levels between 200 cells/μL and 500 cells/μL are pathological and can be used as inclusion criterion for eosinophil targeted therapies [19]. Little is known regarding the meaning and strength of association between blood eosinophilia and presence of eosinophils in different tissue compartments such as the lung.

At birth mice only have a few eosinophils present in the lungs. Directly after birth epithelial stimulation by breathing leads to IL-33 production which stimulates type 2 innate lymphoid cells to produce IL-5. Subsequently, IL-5 recruits eosinophils to the lungs [20,21]. The attracted eosinophils in the lungs are hypothesized to regulate a state of homeostasis and have a protective role in the defence against viruses in the newborn [22].

Eosinophils are absent in tissues that are typically sensitive for allergic inflammation (skin, nose, airways and conjunctiva), whereas they are present in multiple tissues that are rarely affected by allergic inflammation (intestine, adipose tissue, endometrium etc). This finding clearly indicates that the cells are most likely involved in homeostatic functions such as energy balance [23,24] and macrophage functions in the intestine [25]. These more general functions of eosinophils are beyond **the** scope of this short review that will be from now focussed on the lung.

A regulatory homeostatic function of the eosinophil is supported by the finding that *resident* eosinophils in lung tissue of mice prevent the development of a Type 2 response to ovalbumin sensitisation [26]. Importantly, the presence of *inflammatory* eosinophils did not prevent this Type 2 response. The situation in humans remains to be established as the finding of eosinophils in stroma of the lung in healthy individuals remains to be reproduced [27]. Therefore, the finding of two populations in mice was not confirmed in humans and up to now there is no data to support the actual **presence** of rEos and/or iEos in the stroma of human lungs in homeostasis.

Eosinophil activation: a multistep phenomenon (Fig. 1). The eosinophil is one of the most cytotoxic cells in the human body. Therefore, this cell needs to be carefully controlled in a way such that its cytotoxic potential is released just sufficiently to reach its goal, without causing collateral damage by over-activation. This is achieved by a multistep activation mechanism beginning with priming and ending with full-blown activation (degranulation and activation of the respiratory burst) (see Fig. 1). Under homeostatic conditions the cells are refractory to activation requiring high concentrations of cellular activators. The first step towards activation is 'priming' by inflammatory mediators [28]. An eosinophil is primed when a priming agent does not directly activate a cytotoxic mechanism, but enhances a response by a heterologous activator. Priming is typically associated with very subtle changes of inflammatory signals on the cell membrane, such as the expression of conformational epitopes [29–32] or with enhanced functional responses [33,34]. Hereafter, activation of eosinophils depends

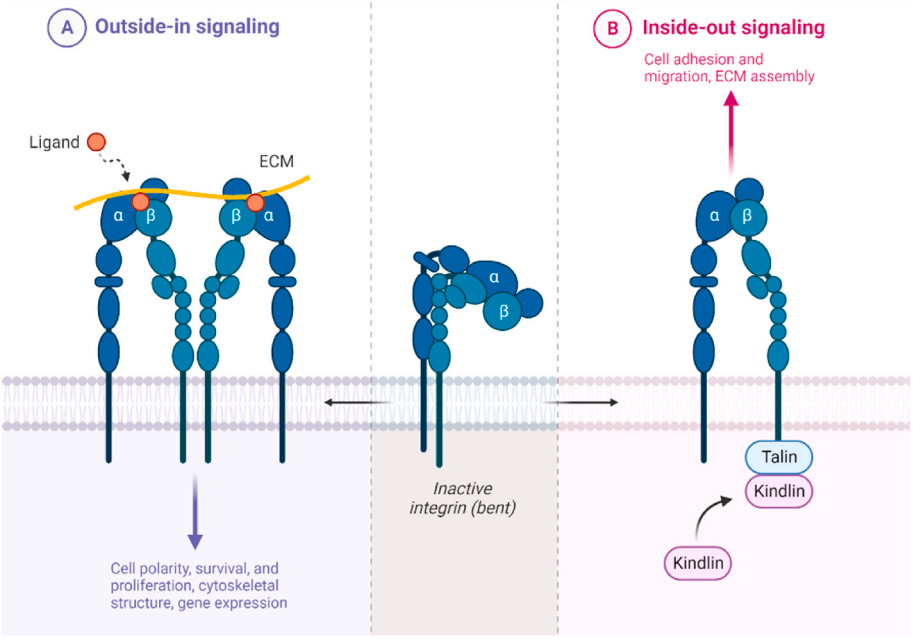

Fig. 2. Outside-in versus inside-out signalling. Adapted from "Outside-in and Inside-out Integrin Signaling Pathways", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

on the type and concentration of activators. Opsonized targets are most **quickly** capable of causing a cytotoxic response [35–37].

1. Local and systemic eosinophil (pre)activation during non-infectious T2-inflammation

Identification of phenotypically distinct eosinophil subsets is important to determine the presence of real subsets differentiated in the bone marrow and/or eosinophils that are activated/instructed by tissue cues outside the bone marrow. In the latter situation, eosinophils change upon interaction with stimuli such as inflammatory mediators and mediators controlling migration from the bone marrow towards the blood stream, and from the blood stream to effector sites. Straightforward up- and downregulation of receptors on the eosinophil surface is one of the features of a changing cell which is dependent of the type and extent of an eosinophil driven inflammatory response. However, more subtle changes in expression of inflammation associated cellular markers occur by a mechanism generally named inside-out control by which receptors/proteins change their confirmation [38–40] (see Fig. 2). This mechanism has been studied mostly in integrin receptors that are also present on granulocytes. Integrins are heterodimeric receptors made up of an alpha chain non-covalently bound to a beta chain. Functionally they are important in cell-to-cell adhesion and adhesion to the extracellular matrix (ECM). On the surface of quiescent, resting cells the function of integrins is low and does not facilitate binding. When the eosinophil encounters inflammatory mediators, these proteins can increase in expression as well as rapidly change their configuration, which is associated with changes in the functionality of the cell. The change facilitates binding to specific ligands, which is important for capturing viruses and/or killing opsonized targets (Fig. 2).

Integrin receptors can either be influenced by extracellular ligands encountered by the cell (outside-in) or by intracellular signalling (inside-out). The ability to bind epitopes depends on 1) affinity influenced by receptor confirmation and concentration of extracellar stimuli and 2) valency which is dependent on receptor clustering on the cell surface.

Two activation epitopes that are only present on eosinophil integrins in their activated configuration have been studied more extensively during health and disease *ex vivo*. These activation epitopes illustrate the importance of receptor confirmation instead of a total number of receptors.

1. Johansson and colleagues [29,30] used an antibody directed against the active form of beta-1 (β1) (CD29, clone N29) integrins to study the activation of eosinophils in asthma. They found relatively high expression of active β1 integrins on eosinophils in peripheral blood of patients with difficult-to-treat type of asthma studied in the Severe Asthma Research Program (SARP). The expression was associated with a high number of eosinophils in these patients. Importantly, the active configuration of the integrins was higher than controls, while the overall expression of integrins did not change that much.
2. Koenderman and colleagues used two phage antibodies to measure the expression of Fcγreceptor II-a (CD32a) in active conformation on eosinophils isolated from peripheral blood after challenge of human airways with allergen [14]. They found that in normal human asthma priming of eosinophils in the peripheral blood is a common feature [32]. In fact, such expression of activation markers might be a predictor of the known success of local treatment (ICS) in the asthmatic lung. Segmental lung allergen challenge can cause this priming state and primed cells are found in the lung of atopic patients after allergen challenge [31,14]. On the other hand in more severe, eosinophilic asthma, peripheral blood eosinophils are refractory to formyl-peptides fMLF and therefore express low levels of active CD32 after stimulation [41].

2. Local and systemic eosinophil (pre)activation: viral infection

Defence against viruses. The beneficial role of eosinophils in the defence against viruses is supported by the protective effect of activated eosinophils in the immune response against respiratory viruses [42]. In a murine model of eosinophilia, IL-5-transgenic mice recover more quickly from an influenza infection compared to wild-type mice with lower eosinophil levels in their peripheral blood [43]. Human eosinophils also have the capability to bind, capture and inactivate viruses [42]. Strikingly, the capacity to bind and capture viruses is lowered in patients with asthma [43]. This is not well understood but it might point at an important mechanism operational in homeostasis. In asthma more eosinophils are present in the airway, lung tissue and peripheral blood yet their status and functional capabilities differ from eosinophils from healthy counterparts. This specific study observed an activation of eosinophils upon binding to the virus. Already activated eosinophils such as found in high concentrations in sputum of asthma patients might not bind and clear viruses as well [44]. This could explain the higher incidence and more severe course of disease of viral infections in patients with asthma as observed in this study.

Eosinophil activation in COVID-19. Many COVID-19 patients present with an eosinopenia [45]. The relevance of this finding is still unclear. However, an absolute eosinopenia proved to be an indicator of severe disease and unfavourable outcome [46,47]. The activation status of the 'remaining' eosinophils was studied and these cells were found to be unresponsive to Formyl-peptide stimulation [48]. The responsive cells are presumed to have migrated to the lung tissue, a phenomenon also seen after allergen challenge [14,49]. Importantly, eosinopenia is not specific for COVID-19 as it also occurs in seasonal influenza, although less frequently [50,51]. One study compared the incidence of eosinopenia (<100 cell/μL) between COVID-19 (60%) and influenza (16%) which implied it is far more common in COVID-19 [52].

Eosinophil activation during other viral infections. Influenza and also other respiratory viruses, most importantly rhinoviruses (RV) and respiratory syncytial virus (RSV) [53], are associated with human asthma [54]. RV infections are clearly associated with exacerbations of allergic asthma [55,56]. Whether RV directly or indirectly influences the eosinophil compartment remains to be established. The situation with RSV is clearer, as a link between eosinophils and the virus was already suggested in 1969, in the early vaccination trials with formaldehyde-inactivated virus that showed altered responses to reinfection with RSV even leading to fatal eosinophilia in the lung in a small group of recipients [57]. The underlying pathogenetic mechanism remains to be elucidated, but RSV can induce activation and survival of eosinophils in vitro [43,58]. In addition, RSV infection in young children *in vivo* is associated with activation of the eosinophil compartment *in vivo* [59,60].

3. Local and systemic eosinophil (pre)activation: severe eosinophilic asthma

Clinically, severe eosinophilic asthma (SEA) often presents with a discordance between symptoms and the severity of eosinophilic inflammation [61]. Patients can have low symptom expression in between severe 'eosinophilic' exacerbations. Autopsy reports from patients with fatal asthma typically describe thickening of smooth muscle layer in combination with plugging of narrowed bronchioles with thick mucus containing eosinophils and neutrophils. Additionally high numbers of eosinophils are found in the mucosa, submucosa and mucus glands [62]. These hallmarks have been known for over 100 years [63]. Importantly also high levels of eosinophil granule contents were found to be present in sputum of patients with SEA, indicating a prior state of activation and degranulation [64]. Among these cytotoxic proteins are Eosinophil Cationic Proteins (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPX/EPO) [65]. In summary, in SEA there is an accumulation of eosinophils within the airways with also high numbers

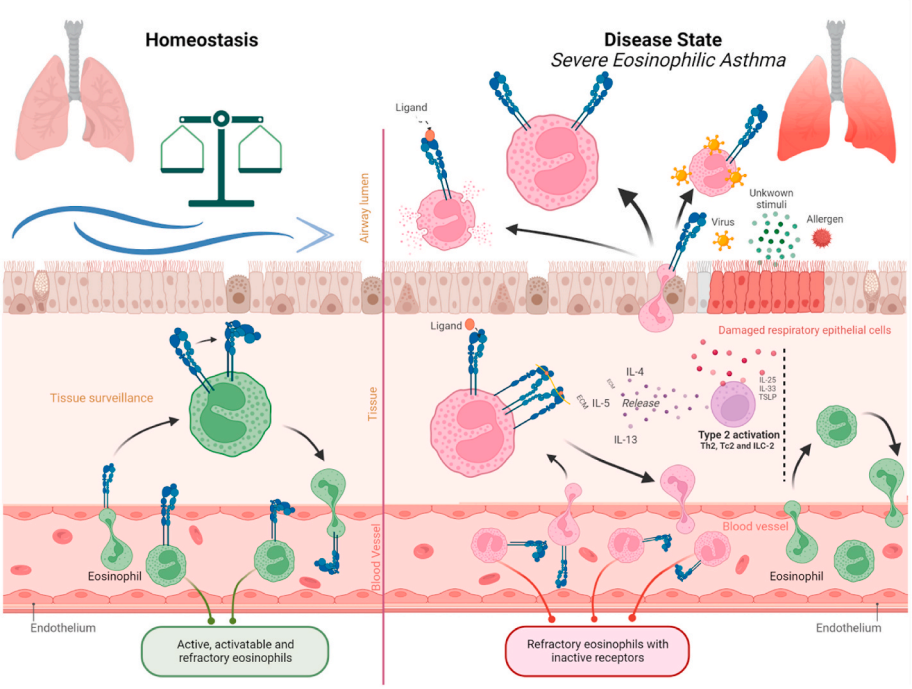

Fig. 3. Homeostatic and pathogenic patterns in eosinophil activation. Created with BioRender.com

of eosinophils present within the airway wall and in lung parenchyma. These eosinophils have different states ranging from resting cells to very active and degranulated apoptotic cells [44].

High numbers of eosinophils are also present in peripheral blood of patients with SEA. The status of these cells ranges from a refractory state to an activated state [66]. The levels of cell-surface proteins on eosinophils help to differentiate between the different states of eosinophils. Specifically expression levels and protein configuration of Fc-receptors and integrins indicate a 'primed', 'active' or 'refractory' status. After allergen challenge there is a gradual increase of priming in blood eosinophils, characterized by the upregulation of active FcγRII [14]. This seems to contradict the abundance of inactive or refractory eosinophils in peripheral blood of patients with SEA with proven airway eosinophilia [44]. A possible explanation for this observation could be that all eosinophils susceptible to priming constantly migrate to the airways resulting in a refractory pool in peripheral blood likely followed by depriming [29,30,14,67]. This is supported by downregulation of other surface proteins such as CD44 a hyaluronan receptor and CD48 in the context of severe asthma [68–70]. CD44 is linked to the movement of eosinophils to the airway in mice after allergen challenge [71] and therefore a lower expression level might indicate transmigration of a subset of eosinophils with higher expression levels towards the allergen-challenged lungs [29,30,14].

The effect of biologics such as anti-IL-5(Rα)(mepolizumab, reslizumab, benralizumab) and anti-IL-13-IL4Rα (dupilumab) on eosinophil priming or activation has been studied to a limited extent [72]. One study describes eosinophil activation into depth in the context of mepolizumab and allergen challenge [73]. In this study mepolizumab lowered eosinophil numbers in pretreated subjects who underwent allergen challenge compared to a challenge within the same subjects without pretreatment. The numbers of eosinophils were lower in blood and airways but their functional phenotype was not altered significantly. Peripheral blood eosinophils expressed less IL-3R and had less mRNA encoding for eosinophil-derived neurotoxin (EDN) compared to the allergen-challenge without mepolizumab pretreatment. Importantly BAL eosinophils had similar expression of CD23, CD44, and CD69, and receptors for the IL-5 family cytokines (IL-5, IL-3, and granulocyte–macrophage colony–stimulating factor) in both groups. These findings lead to the assumption that mepolizumab suppresses eosinophil numbers in peripheral blood and airways, yet does not influence eosinophil function nor their pathological role in severe eosinophilic asthma. Importantly this is supported by real world data coming from the MEX-study, in which the nature of asthma exacerbations in patients on treatment with mepolizumab was determined into much detail. Patients on mepolizumab continued to have eosinophilic exacerbations in ~50% of total. Importantly overall exacerbation frequency was much lower and the nature of exacerbations was milder [74].

The effect of anti-IL-5Rα receptor blockade (benralizumab) on eosinophil phenotypes cannot be studied due to eosinophil depletion. To date, the effect of anti-IL13-IL-4Rα (dupilumab) on eosinophil phenotypes has not been studied. Strikingly, treatment with dupilumab is associated with a transient eosinophilia [75] in peripheral blood and in atopic dermatitis patients also in the conjuctivae [76]. Whether these eosinophils are refractory, primed or activated is yet unclear. Finally, the aforementioned allergen-challenge study by Kelly et al. [73] does provide insight in the mechanism of allergy-induced eosinophilia, but does not answer the question which activation status of eosinophils is present in patients with SEA. It is generally known that allergies are not the driver of the disease in SEA [61]. In summary, little is known regarding the activation status of eosinophils in peripheral blood of patients with SEA treated with biologics.

In conclusion: is eosinophil activation *in vivo* a marker of disease or tissue homeostasis or both? Many studies in the eosinophil field compare cells from peripheral blood with cells in diseased tissue. Differences found in tissue eosinophils are generally interpreted as disease specific phenomena. Unfortunately, an essential control is missing: the eosinophil that extravasates towards the tissue under homeostatic conditions. For neutrophils it has been shown that extravasation from blood to sputum under healthy conditions *per se* is associated with an activated phenotype such as found in diseased conditions [77]. This finding emphasizes the importance to discriminate between homeostatic and pathogenic signals (Fig. 3). Hypotheses regarding tissue eosinophils are better approached by comparing eosinophils obtained from tissue already populated with these cells with eosinophils in the same tissue in context of a disease [8]. In peripheral blood eosinophil activation leads to homing, leaving behind the less activated or refractory cells. This

leads to the counterintuitive finding of very limited activation of eosinophils in peripheral blood in more severe disease, because all activated cells have extravasated into the tissue. A solution could be to study eosinophil phenotypes in peripheral blood in homeostasis and disease over time. It is to be expected that longitudinal data from peripheral blood in patients with type 2 disease will allow identification of subtle changes in eosinophil activation over time with each patient being its own control. This could be an important breakthrough for eosinophilic diseases, because more specific disease patterns can be identified by a very common clinical test: withdrawal of blood and look at quality (eosinophil activation status) in addition to quantity.

In homeostasis, shown at the left side of Fig. 3, eosinophils are recruited into the pulmonary tissue upon activation in the blood stream. After tissue surveillance eosinophils reverse-migrate into the circulation. At the right side of the figure, in a disease state, eosinophils are activated in the blood stream, migrate into the tissue and migrate onwards into the airway lumen executing an effector function (e.g. phagocytosis). Long term pathogenic signaling in severe eosinophilic asthma results in refractory/inactivatable eosinophils in the bloodstream and in high numbers of activated eosinophils in the lung tissue and airway lumen.

CRediT authorship contribution statement

B. Hilvering: Conceptualization, Writing – original draft, contributed equally to the conceptualization of the manuscript, wrote an initial draft of the manuscript. **L. Koenderman:** Conceptualization, Writing – review & editing, Supervision, contributed equally to the conceptualization of the manuscript, contributed content, edited and supervised the following versions of the manuscript, contributed equally to the creation of figures in the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hilvering and Koenderman report relationships with GSK that includes: consulting or advisory. Hilvering reports a relationship with AstraZeneca Medimmune that includes: consulting or advisory.

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