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Shaping eosinophil identity in the tissue contexts of development, homeostasis, and disease

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Abstract

Eosinophils play homeostatic roles in different tissues and are found in several organs at a homeostatic baseline, though their tissue numbers increase significantly in development and disease. The morphological, phenotypical, and functional plasticity of recruited eosinophils are influenced by the dynamic tissue microenvironment changes between homeostatic, morphogenetic, and disease states. Activity of the epithelial-mesenchymal interface, extracellular matrix, hormonal inputs, metabolic state of the environment, as well as epithelial and mesenchymal-derived innate cytokines and growth factors all have the potential to regulate the attraction, retention, *in situ* hematopoiesis, phenotype, and function of eosinophils. This review examines the reciprocal relationship between eosinophils and such tissue factors, specifically addressing: 1) tissue microenvironments associated with the presence and activity of eosinophils; 2) non-immune tissue ligands regulatory for eosinophil accumulation, hematopoiesis, phenotype, and function (with an emphasis on the extracellular matrix and epithelial-mesenchymal interface); 3) the contribution of eosinophils to regulating tissue biology; 4) eosinophil phenotypic heterogeneity in different tissue microenvironments, classifying eosinophils as progenitors, steady state eosinophils, and Type 1 and 2 activated phenotypes. An appreciation of eosinophil regulation by non-immune tissue factors is necessary for completing the picture of eosinophil immune activation and understanding the functional contribution of these cells to development, homeostasis, and disease.

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AUTHOR CONTRIBUTIONS

H.A.-V. and S.B. outlined and structured the concepts covered in this review. All authors participated in writing and editing the manuscript. M.E.C. and S.B. designed and prepared the figures. Final version of the manuscript was approved by S.B.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

Keywords

eosinophils; epithelium; mesenchyme; extracellular matrix; phenotype; *in situ* hematopoiesis; development; homeostasis; asthma; metabolism

Eosinophil recruitment is a hallmark of allergic inflammation, thought to contribute to both the initiation and propagation of tissue damage and remodeling during allergic inflammation. However, it is important to realize that in return, changes in the tissue microenvironment itself may elicit recruitment as well as functional and phenotypic change in immune cells as part of the mechanism to return to tissue homeostatic condition. In agreement with this concept of immune cells as responders to changing tissue environments, studies employing eosinophil-deficient models now demonstrate that eosinophils have multiple novel roles in homeostasis. Eosinophils are found in several organs at normal baseline, assisting in normal tissue processes, and are also recruited to other sites during morphogenesis and repair. Recent studies demonstrate the existence of eosinophil subsets and plasticity in different tissue contexts, reinforcing the significance of change in the eosinophil's immediate environment. Tissue remodeling events during normal morphogenesis and pathogenic remodeling in disease are remarkably similar, which may provide a key to understanding the fundamental nature of tissue programs necessitating eosinophils. Epithelial-derived cytokines and chemokines, increased epithelial shedding and turnover, the extracellular matrix, multiple mucosal ligands such as mucins and sialic acids, mesenchymal stem cell activity, and the metabolic state of the environment are all implicated in the regulation of the attraction, retention, *in situ* hematopoiesis, and function of eosinophils. This review will concisely summarize the current understanding of the two-way communication between tissue and eosinophils, discussing tissue ligands in the framework of biological processes occurring in different tissue states. This review will be divided into four parts: 1) tissue processes that eosinophils associate with in development, homeostasis, and disease; 2) tissue ligands with the potential to engage eosinophils; 3) the potential return contribution of eosinophils in shaping their tissue microenvironments; 4) tissue-based heterogeneity of eosinophil phenotypes and morphology.

Tissue microenvironments associated with increased eosinophil presence and activity

It is now being increasingly recognized that eosinophils populate various tissues in normal development, homeostasis, and disease and that they can occur in both immune and non-immune contexts. Moreover, the same organ may experience influxes of eosinophils in different tissue states, such as development, normal injury repair, or chronic disease. In most homeostatic adult tissues, the numbers of resident eosinophils range from very limited to non-existent. For instance, resident eosinophils in the healthy adult mouse lung average 1.5% of the total $CD45^+$ hematopoietic cell population (1, 2), although the turnover rate and functional significance of such resident populations is not well understood. Eosinophil numbers increase significantly in the following types of tissue microenvironments: *1)* at homeostatic sites where epithelial cell turnover and stem cell activity are high (small intestine, endometrial lining of the uterus, bone marrow, thymus); *2)* during developmental/

morphogenetic events (ductal differentiation of mammary glands (3), development of Peyer's patches (4), postnatal lung development (5), beige fat biogenesis (6–8); *3)* during processes of normal injury repair; *4)* in diseases involving extensive tissue remodeling (helminthic parasite infections, acute lung injury (oxygen stress), fibrosis, cancers, allergic diseases of the gut, skin, and airway, and non-atopic diseases with a significant remodeling component (eosinophilic esophagitis, chronic rhinosinusitis, AERD)) and *5)* endocrinopathies. For details of eosinophil activity in each of these particular scenarios, we would like to refer you to the excellent reviews by James Lee et al. (9, 10), Melo et al. (11), and Rosenberg et al. (12). Instead, we will focus on reviewing fundamental features of the tissue microenvironment common to all these situations and summarize the representative features of tissue physiological states associated with eosinophil presence and activity.

When comparing the different situations in which eosinophils are found, it becomes clear that the processes engaging eosinophils in health and disease are morphogenesis and regeneration, stem cell activity, and changes in the tissue metabolic state. One has to recognize that these three processes are intricately intertwined, as stem cells give rise to differentiated cells and metabolic adjustments are necessary to provide sufficient energy for regeneration processes. As initially postulated by James Lee in the ***LIAR*** hypothesis (9), eosinophils are intrinsically homeostatic cells that are regulators of ***L***ocal ***I***mmunity ***A***nd/or ***R***emodeling/Repair in both health and disease, generally associated with sites characterized by bursts of cell proliferation and death. This notion fits well with an emerging view of Type 2 immunity having evolved as a restorative response to assist in tissue repair and remodeling, an arm of immune response with which eosinophils are associated (13, 14). For example, Huang et al. (15) provide supporting evidence suggesting that Th2 immune responses and homeostatic eosinophil activity may have evolved not to expel parasites but rather to limit inflammation, control tissue glucose uptake, and minimize potential damage to the host (15). Here, we will provide an in-depth view of these processes, focusing on overarching mechanisms associating with eosinophils in all types of the environment.

Upon careful review, the establishment of epithelial-mesenchymal communication emerges as one of the central themes in tissue morphogenesis associated with eosinophils. It also becomes remarkably apparent that the association between the epithelial-mesenchymal interface and eosinophils is conserved between normal morphogenesis (modeling) and disease (remodeling) (Figure 1), involving the same key players (activity of mesenchymal cells and growth factors, modification of the ECM) and an identical set of developmental pathways (WNT, Notch, Hippo, Smoothened, Hedgehog) responsible for epithelial patterning. For example, normal mammary gland development has been described as wound-like in both mice and humans (16, 17). Similarly, morphogenesis of the lung continues during postnatal development, characterized by the deposition of the provisional extracellular matrix, increased mesenchymal-epithelial communication, differentiation of mature stratified epithelium (18, 19), as well as processes associated with the loss of epithelial differentiation in chronic allergic disease (20, 21). The only apparent difference between normal and pathological states of the tissue undergoing remodeling is in *heterochrony*: timing of the onset, offset, and persistence of these signals. In normal development and injury repair, homeostatic modeling programs are perfectly timed, while in

disease they are perpetuated, perhaps associated with a continuous attempt to return tissue to a homeostatic state.

In allergic disease, the notion of eosinophils as direct responders to changing states of the tissue environment is rarely exercised. In the subsequent sections of this review, we will address the two-way communication between eosinophils and tissue in homeostasis, development, and disease, with a focus on the tissue ligands acting on eosinophils as well as eosinophil products with an impact on tissue biological processes. Acknowledging this communication is a significant step towards understanding how tissue microenvironments shape eosinophil identity and function in disease and how these cells could be targeted therapeutically in highly heterogeneous allergic disease scenarios.

Tissue ligands regulatory for eosinophil accumulation, phenotype, and function

The coordinated behavior of multiple processes is necessary to achieve tissue homeostasis, such as well-tuned epithelial-mesenchymal communication, the controlled deposition of extracellular matrix, the differentiation of specialized cells, proteoglycan, aminoglycan, and mucin synthesis, proper regulatory input of hormones and metabolites, and balanced activity of the innate immune system. Here, we will review key tissue players and ligands that both represent these processes and are known to elicit an eosinophil response (summarized in Figures 2 and 3).

Epithelial-mesenchymal interface

The LIAR hypothesis postulates that eosinophils are attracted to sites of high stem cell activity that accompany all tissue generation and regeneration events. The epithelial-mesenchymal transition (EMT) has recently been implicated in multiple allergic diseases as a mechanism causative of mucosal barrier disruption (20, 22, 23). In homeostatic conditions, EMT occurs as a mechanism of maintaining epithelial barrier integrity by potentiating the mesenchymal unit to create new layers of differentiated epithelial cells (18). At sites such as the intestine, epithelial shedding rates are high and mesenchymal-epithelial turnover is much higher. Interestingly, eosinophils frequent such sites in the small intestine and colon while being much less frequently found in organs such as the lung, where epithelial turnover is much slower. Chemokines, growth factors, and secreted ECM proteoglycans and morphogenetic ligands are all mesenchymal-secreted ligands that can engage eosinophils. Fibroblasts are frequently seen as central to eosinophil recruitment as they can be a prominent source of eotaxins and growth factors (24, 25). Activation of the Wnt (26), Hippo (27), Notch/Jagged (28) and Hedgehog (29) developmental pathways is necessary for epithelial development and patterning and is strongly associated with epithelial remodeling. Importantly, EMT-related morphogenetic ligands such as WNTs and Hedgehog proteins can be secreted by remodeling tissue and can directly engage eosinophils and other immune cells (29–31). Moreover, the mesenchymal compartment and myofibroblasts in particular have the ability to secrete multiple provisional ECM proteins with multiple regulatory effects on immune cells (32–34).

Extracellular matrix

The ECM is an intricate network of macromolecules that forms the 'scaffolding' of the airways and other tissues. This scaffolding not only acts as a mechanical support that plays a crucial role in the maintenance of airway function and structure but is also a dynamic and complex signaling network that has the potential to regulate the function of multiple structural and immune cells, including migration, proliferation, and differentiation via integrin and toll-like receptor engagement (35). The ECM is critical for guiding development and repair at the mesenchymal-epithelial interface (36). ECM composition is not uniform along the epithelial barrier; it is a dynamic structure exhibiting spatio-temporal heterogeneity depending on the needs of the surrounding tissue. The ECM changes dramatically during development and disease (37, 38). The transient deposition of the provisional, “immature” matrix, characterized by the secretion of proteoglycans such as tenascins, periostin, mindin, hyaluronan, and versican (39, 40), is critical for epithelial differentiation (41–43). This is exemplified by the unique deposition of Tenascin-C during development in areas undergoing active epithelial differentiation (41, 44, 45). Eosinophil numbers in the asthmatic airways correlate with ECM markers such as periostin and the thickening of the reticular basement membrane (RBM), characterized by the deposition of specific ECM proteins (46–48). The basement membrane thickness can range from 7 to 23 μm in subjects who have asthma, while it is only 4 to 5 μm in healthy individuals (49). Airway eosinophils in asthma exhibit a hyperadhesive phenotype towards provisional ECM and are well equipped to interact with its components via expression of specific integrins (CD11c, CD11b, beta 5 integrins) and TLRs (TLR4, in particular) (50–52). An interesting implication of the eosinophil-ECM association in disease is that an EMT-perpetuated imbalance between ECM component synthesis and degradation may represent a persistent tissue-based driver of eosinophilia. This idea remains to be tested. Another interesting implication of changing ECM dynamics in development and disease is a potential regulatory role for eosinophil bone marrow or *in situ* hematopoiesis. For example, the extracellular matrix establishes hematopoietic gradients in the stromal environment of murine bone marrow and is critical for sustaining hematopoiesis (53). Mice lacking TNC show reduced colony-forming capacity and hematopoietic cell production (54). Hyaluronic acid scaffolds are sufficient to maintain long-term cultures of CD34+ hematopoietic cells obtained from human cord blood (55). Periostin is highly expressed, specifically in fibroblasts that support hematopoiesis (56).

Mucosal-derived factors

The trafficking of eosinophils to mucosal tissue during homeostasis and disease is regulated by CCL11 and Th2 cytokines. Epithelial-derived eotaxins are fundamental signals that regulate eosinophil homing to the GI tract in mice (57). CCL11 is required for the baseline level of tissue eosinophils (58). Mice deficient in CCR3 or CCL11 have defective tissue homing of eosinophils to the lamina propria of the GI tract (59). In addition to CCL11, the Th2 cytokines IL-5 and IL-13 are also critical in sustaining the GI trafficking of eosinophils during homeostasis (60). Eosinophils are also regulated by the epithelial-derived innate cytokines TSLP and IL-33 that both directly activate eosinophils and promote their recruitment via Th2 response amplification. TSLP is an IL-2 family member that primes Th2 responses via the activation of dendritic cells (DCs) (61) and basophils (62). IL-33 is an IL-1

cytokine family member present in the nucleus of structural cells such as fibroblasts, epithelial cells, and endothelial cells and is released during inflammation and cellular distress (63). TSLP prevents the apoptosis of eosinophils by direct activation of the TSLPR present on eosinophils (64). Stolarski et al. (65) showed that IL-33/ST2 signaling activates murine airway eosinophils, and other work showed that the IL-33 stimulation of eosinophils induces marked gene expression and the release of chemokines and cytokines such as IL-4 and IL-13 (66, 67). IL-33 also increases the activity and survival of human eosinophils (68) and, in a murine adoptive transfer model system, provides a survival advantage that allows for greater pulmonary trafficking (69). Type 2 lymphoid cells (ILC2) control the local accumulation of mature eosinophils in peripheral tissues at baseline (70) and development (5). Among other mucosal factors, the glycoprotein lactoferrin secreted by glandular epithelial cells stimulates eosinophil activation (71). Surfactant protein SP-D (a C-type lectin) is a limiting factor for eosinophils in the airway (72). Similarly, the engagement of another lectin, Siglec-F in mice and Siglec-8 in humans, by engaging corresponding ligands (such as 6'-sulfo-sialyl Lewis X glycoproteins for both, and glycans on Muc5b for Siglec-F) activates a pro-apoptotic response, especially for human eosinophils (73, 74).

Metabolism and hormones

Local tissue processes are also governed by local and system-level changes in metabolism (conceptualized in Figure 3). There is mounting evidence that eosinophils play a crucial role in metabolic homeostasis and also respond to changes in metabolism. In mouse models of high-fat diet-induced obesity, eosinophils promote insulin sensitivity and are associated with better glucose responses, more lean body mass, and higher energy expenditures (75, 76). Wu et al. (75) first linked the recruitment of eosinophils to the beneficial process of fat beiging and the control of metabolism and glucose homeostasis. The authors showed that eosinophils are the main source of interleukin (IL)-4 in white adipose tissue (WAT), which was necessary for the induction of alternatively activated macrophages (AAMs). Eosinophils were also found to associate with insulin resistance and β-cell dysfunction in pre-diabetic subjects (77), as well as corticoadrenal insufficiency (78). Eosinophils are linked to the development of Type I diabetes and express high levels of myeloid alpha-defensins and myeloperoxidase in patients with type 1 diabetes mellitus (79). Remarkably, eosinophils express multiple hormone receptors, implicating that their activity in peripheral tissues may be subject to systemic-level regulation by the endocrine system. Human tissue eosinophils express leptin surface receptors, and leptin delays the apoptosis of mature eosinophils *in vitro* (80). Similarly, the estrogen receptor (GPER) is expressed on human eosinophils, which also inhibits eosinophil apoptosis (81). Human eosinophils also express retinoic acid receptors (RARs); retinoic acids are potent inhibitors of human eosinophil apoptosis (82) and upregulate eosinophil expression of CCR3 (83). Moreover, human eosinophils have been shown to express multiple prostaglandin receptors (84) and GPR120, a G protein-coupled receptor for long-chain fatty acids (85). The stimulation of eosinophils with a synthetic GPR120 agonist led to the increased expression of IL-4 and the inhibition of apoptosis. These findings suggest that eosinophils might function as nutrient sensors (85). Finally, there is a strong link between eosinophils and the arachidonic acid metabolism. 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), a 5-lipoxygenase product, has powerful eosinophil chemotactic activity (86, 87). In addition, 5-oxo-ETE can induce the expression

and secretion of matrix-metallopeptidase-9 and urokinase-type plasminogen activator receptor (88, 89), which results in the degradation of matrix components. Therefore, 5-oxo-ETE also promotes the infiltration of eosinophils into tissues. In turn, eosinophils residing in the colonic mucosa have been shown to mediate inflammation resolution by expressing 12/15 lipoxygenase products (resolvins and protectins) in experimental models of colitis and self-resolving acute peritonitis (90, 91).

What do eosinophils contribute back to their tissue environments?

As illustrated above, there are multiple tissue and metabolic factors that are known to modulate eosinophil phenotype and function. However, it is much less clear which processes and morphogenetic events depend on eosinophil presence in tissues, in part due to the complexity of *in vivo* environments and redundant contribution (frequently compensatory) by multiple cell types. In general, eosinophils are thought of as contributors to allergic inflammatory processes and tissue remodeling/fibrosis in asthma (92–96).

The development of constitutive eosinophil knockout mice (PHIL) and inducible eosinophil knockout mice (iPHIL) by Jamie Lee labs allowed us to better understand the tissue roles of eosinophils in development and disease, which in many cases produced surprising results. Contrary to the view of eosinophils as destructive effector cells, eosinophils were found to be necessary for the resolution of inflammation, tissue repair, and the return of lung tissue to homeostasis in a chronic model of asthma, as PHIL mice showed a lack of resolution (97). Interestingly, despite the strong association with the postnatal lung and mammary gland development, eosinophil-deficient mice developed normally with fully functional lungs and mammary ducts and exhibited an overall lack of the developmental phenotype (98). This is not entirely surprising, as many molecules involved in postnatal development (as opposed to embryonic) do not exhibit developmental phenotypes in knockout mice, among them important factors such as IL-33 and Tenascin-C (5, 99). However, by contrasting the gene expression profiles of PHIL mice with wild type controls, we were able to identify a more subtle phenotype, which consisted of the downregulation of mesenchymal genes (*Nes*, *Smo*, *Vim*) and changes in ECM genes (*Spon2*, *Adam33*) not obvious from a histological examination (unpublished observation). We hypothesize that a lack of eosinophils alters tissue ECM architecture and mesenchymal responses, which may lead to aberrant repair and resolution responses when tissue homeostasis is disrupted.

Eosinophils are well equipped to modify their immediate tissue environment. Eosinophils were originally shown to be active players in fibrosis by their capacity to store and release the most potent pro-fibrogenic factor transforming growth factor (TGFβ) (100, 101). TGFβ is generally considered anti-inflammatory; its levels are increased (33) in the bronchoalveolar lavage of patients with allergic asthma and correlates well with airway and parenchymal remodeling (102). When added to fibroblasts *in vitro*, eosinophils stimulate fibroblast proliferation, ECM synthesis, and lattice contraction mostly by the release of TGFβ and IL-1β *in vitro* (96, 103). Both *in vitro* and *in vivo*, eosinophils have been shown to promote airway remodeling by inducing myofibroblast differentiation and ECM deposition (50). Eosinophils can modulate myofibroblasts by the release of growth factors such as fibroblast growth factor (FGF2), nerve growth factor (NGF), and vascular

endothelial growth factor (VEGF) (93). VEGF expression has been demonstrated in asthmatic airways where, in addition to angiogenesis, it is thought to have a role in airway remodeling (104). TGFβ and eosinophils dramatically induce Tenascin-C mRNA and protein expression in nasal epithelial cells. The effect of eosinophils could be inhibited partly by a neutralizing antibody to TGFβ (105). Eosinophils store and release MMP-9, which is important for ECM modification and migration through the basement membrane (106). In fact, the main source of MMP-9 in asthmatic airways is believed to be eosinophils (107). Tenascin-C induces eosinophil MMP-9 expression directly and by collaboration with TGFβ (108). Many of the effects of IL-13 may be mediated by the metalloproteinases. IL-13 overexpression is mediated by TGFβ, and TGFβ activation is MMP-9 dependent. It is possible, therefore, that MMP-9 could be a key molecule proximal to the IL-13-mediated signaling pathway (109). In addition to classical pro-fibrotic factors, some eosinophil cationic proteins were found to be involved in fibrosis (110). Eosinophil cationic protein (ECP) stimulates TGFβ release by human lung fibroblasts (111). Eosinophil major basic protein (MBP) interacts in a synergistic fashion with rIL-1-α or TGFβ to augment fibroblast IL-6-type cytokine production (112). MBP can also activate the synthesis of remodeling factors by airway epithelial cells (113). Moreover, eosinophils contain heparanase, a pivotal enzyme for ECM degradation, and eosinophil MBP was identified as heparanase-inhibiting protein (114). TGFβ release by eosinophils plays another important role in asthma, as TGFβ1 has been shown to induce airway smooth muscle (ASM) cell growth and is a key mediator involved in the tissue remodeling of the asthmatic lung (115). ASM cells also have an effect on eosinophils by stimulating eosinophil differentiation. Both IL-5 and GM-CSF are suggested as the key factors produced by ASM cells that promote this process (116).

Eosinophils exhibit a remarkable functional association with the tissue plasminogen system. CCL11 was shown to promote eosinophil transmigration specifically via the activation of the plasminogen-plasmin system (117). A new study by Uderhardt et al. (118) shows that eosinophils contribute to intravascular thrombosis by exhibiting a strong endogenous thrombin-generation capacity. It relies on enzymatic generation and active provision of a procoagulant phospholipid surface enriched in 12/15-LO-derived hydroxyeicosatetraenoic acid-phosphatidylethanolamines (118). Eosinophils were also described to accumulate in human thrombi (119) and reported to serve as a major source of Tissue Factor (thromboplastin) (120). Importantly, the plasminogen system not only controls blood clotting but also is involved in epithelial remodeling. Hara et al. (121) showed that eosinophils play an inhibitory role on cell surface plasmin generation by bronchial epithelial cells by means of the up-regulation of PAI-1 expression induced by TGFβ. Therefore, the accumulation of eosinophils in bronchial walls may directly promote fibrin deposition and bronchial tissue repair/remodeling in asthma through this protease network (121).

Eosinophils directly interact with epithelial cells as well. Eosinophil-epithelial interactions significantly stimulate the secretion of MUC5AC, PDGF-AB, VEGF, TGF-β1, and IL-8 from cultured NCI-H292 epithelial cells (122). Neutralizing antibodies directed against amphiregulin, as well as pan-metalloproteinase inhibitor GM6001 inhibited the coculture-induced secretion of MUC5AC (122). Human eosinophils produce and release amphiregulin, which is typical for many Type 2 immune cells interacting with the epithelium (123).

Eosinophils are emerging as potentially critical regulators of metabolic homeostasis (85, 124–127). In murine models, eosinophils present in visceral adipose tissue secrete IL-4 (75) that causes subcutaneous white adipose tissue macrophages to polarize toward the alternatively activated phenotype (128). Eosinophils also promote catecholamine production via tyrosine hydroxylase (6), which causes the development of beige fat that ameliorates obesity-induced metabolic changes. Human eosinophils are an abundant source of 15-Lypoxygenase (129). Very recently, Withers et al. (130) identified eosinophils as a novel source of tissue adiponectin. They conclude that eosinophils play a key role in the regulation of the normal function of vasculature and perivascular adipose tissue.

Overall, eosinophil regulatory roles in the tissue appear to center on promoting the tissue-restorative activity of mesenchymal cells, facilitating ECM scaffold deposition (and possibly, subsequent removal) and maintenance of tissue and systemic metabolic homeostasis. Eosinophils may also directly promote epithelial differentiation via the release of amphiregulin and growth factors, as well as by improving insulin sensitivity necessary for the proper guidance of epithelial differentiation.

Shaping eosinophil phenotype and morphology in heterogeneous tissue contexts

Surprisingly, unlike most other immune system cell types, eosinophils are frequently described as terminally differentiated cells uniform in phenotype and function. However, this view of the eosinophil is rapidly changing. Multiple marker-, morphology-, and function-based eosinophil subtypes are now being identified in various immune and non-immune contexts. Upon review of the literature, it is becoming apparent that a few unifying themes determine eosinophil tissue phenotype: (1) *Maturation*. Eosinophils can be found in many organs and tissue contexts in different states along their differentiation continuum. There is plenty of evidence that eosinophils can traffic to tissues in immature states or even develop locally in the tissue via *in situ* hematopoiesis (131); (2) *Organ location*. For example, in steady state, intestinal resident eosinophils are phenotypically different from resident lung eosinophils (132–134); (3) *Morphogenetic activity of the tissue.* Regardless of organ location, eosinophils are shaped by the morphogenetic plasticity of their environment. The lung perfectly exemplifies this scenario. The lung is characterized by on-going airway morphogenesis during postnatal development, then achieves steady state in the adult homeostatic state, and undergoes active remodeling reminiscent of development in chronic allergic asthma. Eosinophils are found in very low numbers at steady state but those numbers significantly increase during development and disease. Moreover, developmental and disease eosinophils are phenotypically distinct from true resident cells in homeostasis (1, 2). Analogous to this, the homeostatic intestine undergoes active epithelial turnover, which also happens at a much slower rate in the steady state lung. In support of a tissue state-driven phenotype, intestinal resident eosinophils constitutively express CD11c, Ly6G, and CD44, which are only expressed in subsets of lung eosinophils during development and epithelial remodeling (132); (4) *Location within tissue*. In allergic murine models, eosinophils frequently exhibit bimodal and compartment-specific phenotypes (135). We previously demonstrated that upon their transition to the airway, eosinophils upregulate

levels of Siglec-F and CD11c, which is not typical of interstitial eosinophils (1). Such phenotypic transition is also associated with changes in eosinophil morphology, where vacuolarization and high segmentation of the nucleus are typical of airway luminal eosinophils (1); (5) *Immune tissue microenvironment*. Immune drivers of the eosinophil phenotype are reviewed elsewhere. In general, Type 2 immunity aligns well with epithelial morphogenesis and repair/resolution, while Type 1 immune activation is characteristic of defense responses not involving extensive epithelial remodeling.

Along with the discovery of heterogeneous phenotypes and morphology in various biological scenarios, eosinophil classification terminology used in publications and conference talks is also becoming more complex. On one hand, multiple terms are given to eosinophil sub-phenotypes found in similar functional contexts. On the other hand, homeostatic eosinophils that could be described with an over-arching term (for instance, *hEos*) are represented by distinct homeostatic sub-phenotypes, depending on the tissue and functional process context. For example, the term “resident eosinophils” (*rEos*) could be referring to an eosinophil exhibiting one phenotype in the steady state lung but a completely different phenotype in the homeostatic intestine where there is high epithelial turnover. Likewise, the term “developmental eosinophils” (*devEos*) could refer to an eosinophil present in the lung during postnatal development that is clearly homeostatic in nature, but the eosinophil could phenotypically resemble an inflammatory eosinophil ($rEos_i$ and *iEos*) that is very different from the true resident eosinophils at steady state. Here, for the ease of interpretation, we will succinctly review the phenotyping literature in the framework of a tissue-based classification of eosinophils, which should also align with the immune-based classification, as specific immune responses associate with specific tissue processes. We will describe murine eosinophil sub-phenotypes as falling within one of these four tissue-based categories: (1) **EoP**. Immature eosinophils recruited as precursors or undergoing *in situ* hematopoiesis; (2) **Steady state**. True tissue residents in morphogenetically quiescent tissues; (3) **Type 1.** Typically interstitial (stromal in general) in acute inflammatory, innate defense, and transient morphogenetic contexts; (4) **Type 2**. Eosinophils associated with a Type 2 immune response, typically found in epithelial contexts (Figure 4).

EoP eosinophils

A growing body of evidence suggests that *in situ* hematopoiesis is a conserved mechanism of the innate immune system that significantly contributes to the development of immunity at mucosal sites (131). Interestingly, eosinophil progenitors can be found alongside mature cells during allergic inflammation (128). In mice, eosinophil progenitors (EoP) are typically described as expressing CD34, IL-5Rα, and Siglec-F, with weaker eosin staining (136). Recently, it was shown that eosinophil precursors and progenitors express ST2 and TSLPR, the receptors for IL-33 and TSLP, which determine IL-5Rα expression and regulate eosinophil progenitor homing and hematopoietic decisions (136, 137). CD34 eosinophil precursors also express functional TLR4 with the potential to influence hematopoietic decisions (138). Importantly, TLR4 stimulation of human HSCs yields higher numbers of TSLPR+ cells in asthmatics compared with healthy subjects (137, 139). It is still unknown to what extent *in situ* hematopoiesis contributes to the generation of eosinophils at tissue sites as opposed to the recruitment of eosinophils from bone marrow and circulation.

Steady state eosinophils

Resident eosinophils in true steady state can be found only in the parenchyma of homeostatic adult lung mouse tissue. These cells are characterized by having very limited numbers in the lung (1–2% of all CD45+ hematopoietic cells in murine lung) and “donut-shape” non-segmented nuclear morphology (1, 2). Phenotypically, in mouse lungs, these cells express intermediate levels of Siglec-F (1), low levels of CD101 and CD62L, and lack expression of CD11c or any other marker typically associated with tissue-activated status (2). Though recently characterized in the lung, it is not yet characterized whether eosinophils of this phenotype can be found in other organs with little morphogenetic activity. Although intestinal, uterine, and skin eosinophils are also resident and homeostatic, their phenotype and morphology is more characteristic of Type 1 activated eosinophils. This could be due to the fact that portions of the GI tract and uterus are more active in homeostasis, constantly undergoing active epithelial shedding and self-renewal.

Type 1 eosinophils

These eosinophils feature markers of steady state eosinophils ($CD101^{low}$, CD62L, Siglec-F^{med}), but exhibit segmented nuclear morphology more typical of activated eosinophils, although they lack the distinct vacuolarization of Type 2 eosinophils (1). In homeostasis, this phenotype and morphology is typical for postnatal mouse lung development (unpublished observations) and postnatal mammary gland development (140). In development, these cells can be found in the stroma surrounding epithelial cells during branching morphogenesis events (140). In adult murine homeostatic tissues, eosinophils with this morphology can be found in the stroma of the uterus at estrus under the influence of estrogen-regulated eotaxin synthesis (141) and in the lamina propria of the jejunum (58). In models of allergic inflammation, Type 1 eosinophils are seen as a minor population of eosinophils (described as $rEos_i$ by Mesnil et al.) (2). In a kinetic murine model of asthma, we demonstrated that the expansion of Type 1 eosinophils coincides with an acute neutrophil influx (associated with Type I cytokine expression) into allergen-challenged lungs, is specific to the interstitium, and is gradually replaced by eosinophils acquiring a Type 2 phenotype and migrating to the airway lumens (1). Analogous to eosinophils, alveolar macrophages were recently found to exhibit a bimodal Siglec-F^{low} and Siglec-F^{high} phenotype (142). Specifically, Siglec-F^{low} monocyte-derived alveolar macrophages were found to drive fibrosis in bleomycin injury models (142). It remains to be determined whether Type 1 eosinophils also play a role in fibrosis and various cancers (143). Interestingly, a subset of CD11c− cells with Type 1-characteristic morphology in allergen-challenged mice expresses high levels of GR1 (144), which could indicate their more recent hematopoietic origin. It is yet to be determined whether Type 1 eosinophils are generated from steady state cells or HSC precursors, or whether these cells are recruited in this state from circulation.

Type 2 eosinophils

The best distinguishing characteristic of these cells in homeostasis (lung, thymus, Peyer’s patches) and mouse models of disease (asthma, chronic colitis) is the high expression of Siglec-F (1, 145, 146) and a very distinct morphology with highly segmented nuclei and presence of vacuoles (1). These cells are also characterized by the acquisition of CD11c (1),

higher expression of CD101, and lack of CD62L (2). Type 2 eosinophils closely associate with the epithelium, are found on the luminal surface of the airway and intestine, and can be readily extracted from bronchoalveolar lavages in mouse models (1). Cells with this phenotype can also be found in lung lavages during postnatal development (our unpublished observations).

Different eosinophil subsets may co-exist and perform different functions in the same tissue. For example, although speculative at this moment, based on their location and association with immune and morphogenetic environments, Type 1 eosinophils may preferentially interact with fibroblasts and assist in building ECM scaffolds, while Type 2 eosinophils may directly interact with the epithelium and participate in scaffold removal and resolution of repair. Future studies employing single cell sequencing approaches and careful functional validation in relevant biological contexts will be needed to resolve origins and functions of different eosinophil subsets.

Targeting eosinophils in allergic disease

In situ proliferation of eosinophils or the recruitment of these cells to sites of inflammation is the target of several biologics (Benralizumab, Mepolizumab, Reslizumab). These biologics target either IL-5 or IL-5Rα, with the intent of inhibiting or ablating eosinophil survival and proliferation. Although these biologics successfully reduce blood and sputum eosinophil levels, the subtype of eosinophils targeted remains undefined and may be limited. For example, several studies in mice suggest that although IL-5 is highly contributory to eosinophil development, it is not critical for their tissue expansion or survival. Mesnil and colleagues (2) demonstrated that IL-5-independent eosinophils exist in the lung (described as steady state eosinophils). Moreover, IL-5 deficient mice still produce basal levels of functionally competent eosinophils which are comparable to wild type mice in the blood, but cannot elicit pronounced eosinophilia in response to inflammatory stimulation following an aeroallergen challenge (147, 148). Additionally, mepolizumab attenuates airway eosinophil numbers in asthma but not their functional phenotype (149). As such, targeting IL-5 or IL-5Rα may reduce the inflammatory state eosinophils (Type 1 and Type 2) but fail to have any effect on tissue progenitor and resident eosinophil populations. In clinical trials of asthma, eosinophil depletion strategies that target IL-5 (mepolizumab) or IL-5Rα (benralizumab) achieve only partial success, which emphasizes the need to recognize the heterogeneity of asthma and identify the specific patient populations where targeting eosinophils can be effective (150). In eosinophilic esophagitis, mepolizumab and reslizumab, two anti-IL-5 antibodies, resulted in the reduction of esophageal tissue and blood eosinophils in children and adults but showed no significant reduction in symptoms (151). This brings up a complicated issue of addressing eosinophil causality in development and the progression of allergic disease. If eosinophils are to be viewed as only responders to underlying causes of Th2 immune response (i.e., cells assisting in the restoration of a tissue homeostatic state), depletion of these cells may not be a sufficient therapeutic measure for allergic disease. Significant promise for future targeting of eosinophils in disease may lie in understanding the homeostatic versus pathogenic activation of eosinophils. Identifying novel targets and the processes underlying Th2 responses at the tissue level is equally important,

among them underappreciated are changes in extracellular matrix and metabolic tissue microenvironment with regulatory activity for eosinophil phenotype and function.

Summary

Placing eosinophils within heterogeneous tissue contexts and acknowledging the significant regulatory potential of tissue factors supports the view of eosinophils as inherently homeostatic cells driven by complex immune and non-immune changes in their immediate tissue microenvironment. The epithelial-mesenchymal interface, extracellular matrix, and metabolism are emerging as novel tissue regulators of eosinophil biology. Understanding eosinophil-tissue interactions in homeostasis will be beneficial to identifying the tissue processes driving eosinophils in disease as well as understanding the return contribution of these cells to disease pathogenesis. Eosinophil phenotyping and classification is an emerging area in eosinophil research with many future challenges. Here, we demonstrate the diversity and complexity of the actual eosinophil tissue microenvironment, where each component has the potential to influence eosinophil phenotype and function. Addressing immune activation alone is not sufficient to explain disease-relevant eosinophil biology. To fully understand eosinophil heterogeneity within actual biological *in vivo* contexts, we need to reconcile the differential activation of eosinophils by the immune system with tissue-driven regulation. Single cell sequencing, novel *in situ* hybridization approaches, context-relevant functional assays, and lineage tracing studies will likely significantly advance our understanding of the true nature of these cells in heterogeneous tissue and immune contexts of development, homeostasis, and disease.

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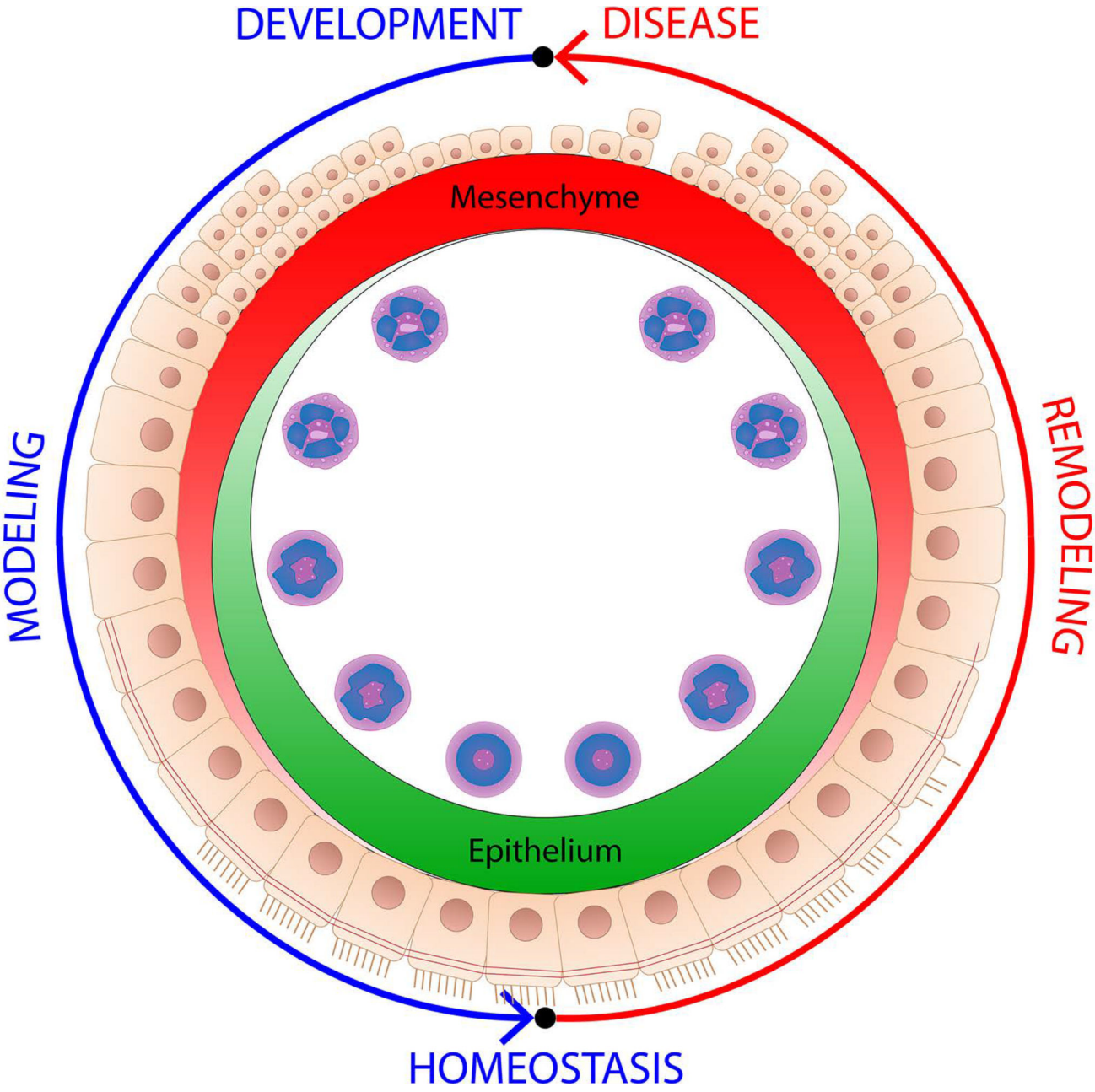

Figure 1.

Eosinophils associate with transient epithelial differentiation (modeling) events in development and persistent (remodeling) loss of differentiation in allergic disease. The two processes are analogous. The only apparent difference between the normal and pathological states of tissue undergoing remodeling is in heterochrony: the timing of onset, offset, and persistence of these signals. In normal development and injury repair, homeostatic modeling programs are perfectly timed while in disease they are perpetuated, perhaps associated with a continuous attempt to return the tissue to its homeostatic state. Changes in eosinophil phenotype typically associate with morphogenetic changes in tissue environment.

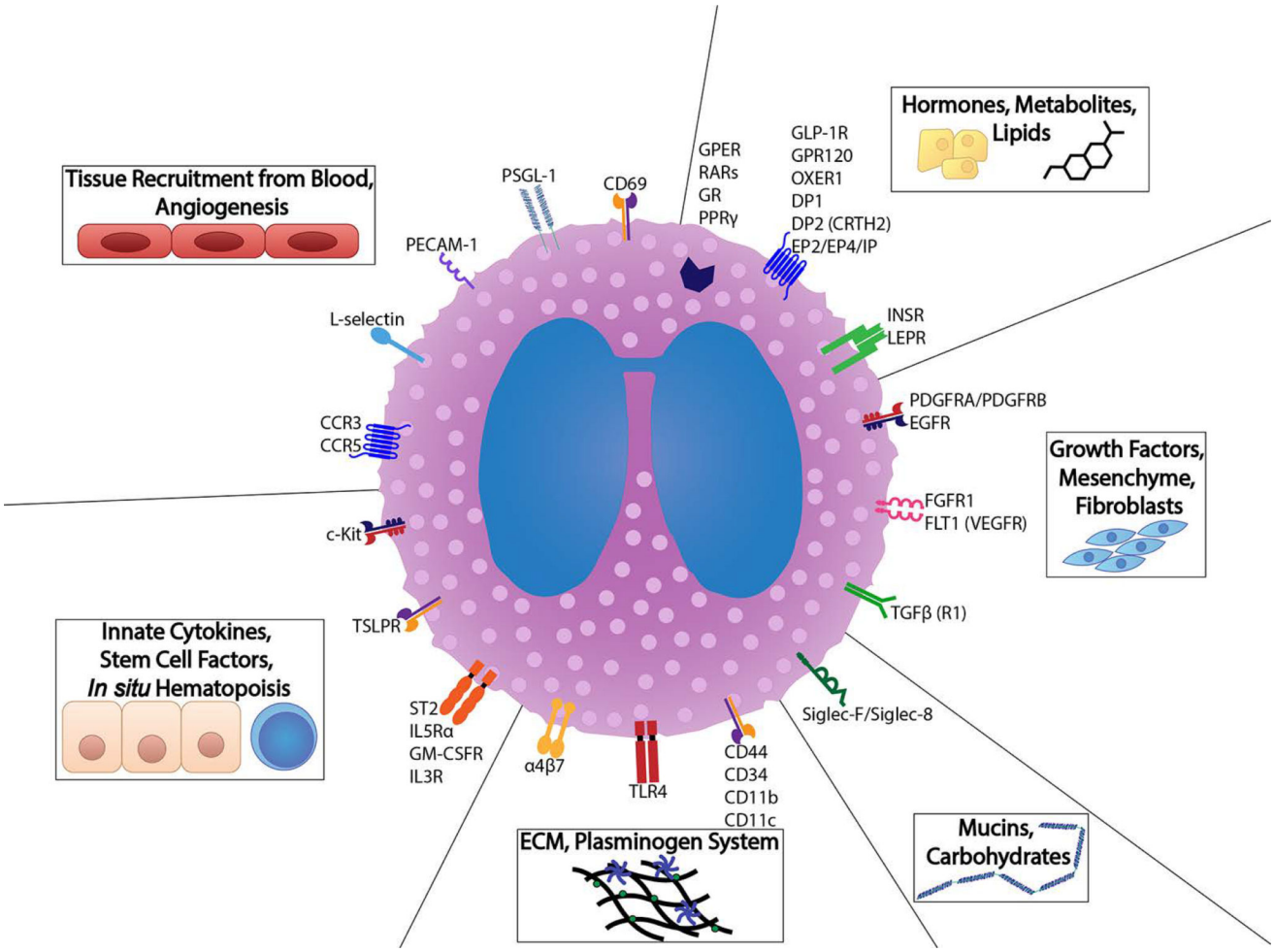

Figure 2.

Eosinophils are well equipped to interact with all components of their tissue microenvironment. Representative receptors are illustrated that correspond to the diverse tissue ligands and cellular processes encountered both by mature and immature eosinophils in the contexts of development, homeostasis, and disease.

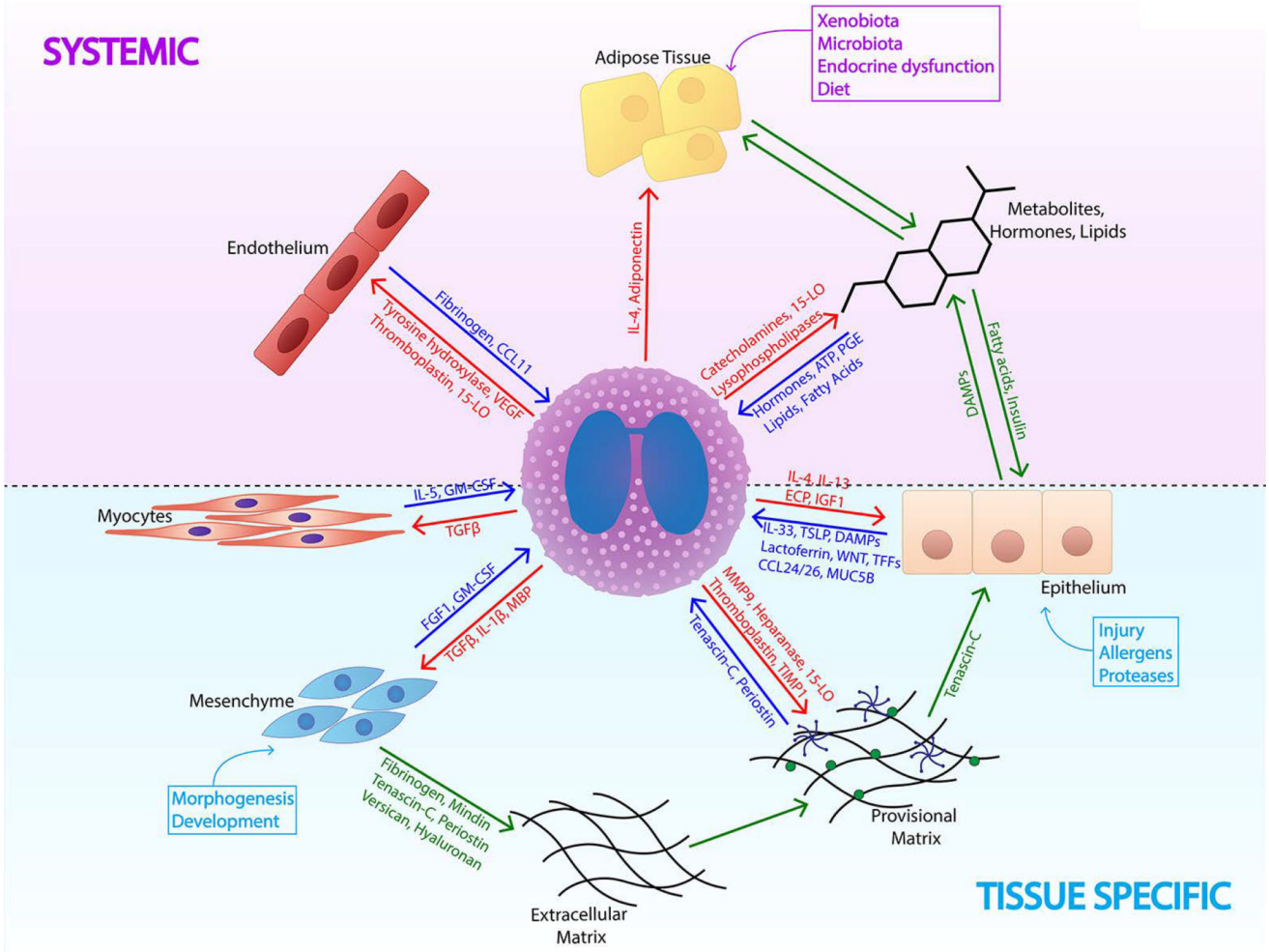

Figure 3.

Eosinophil in the context of its immediate non-immune tissue microenvironment. Eosinophils are both affected by and affect their tissue environments. Eosinophil-tissue interactions are governed both on the systemic level by changes in nutrient availability, hormones, lipids, and overall metabolism (purple) and by tissue-specific processes, such as activation of the epithelial-mesenchymal interface and ECM deposition (light blue). During such activation of tissue programs, eosinophils receive multiple tissue inputs such as alarmins, DAMPs, ECM molecules, lipids, hormones, and metabolites (dark blue arrows). They respond to these cues by releasing products (red arrows) in an effort to return the tissue to homeostatic conditions. Balanced communication of different tissue components and systemic factors (green arrows) is necessary to maintain peripheral tissue homeostasis.

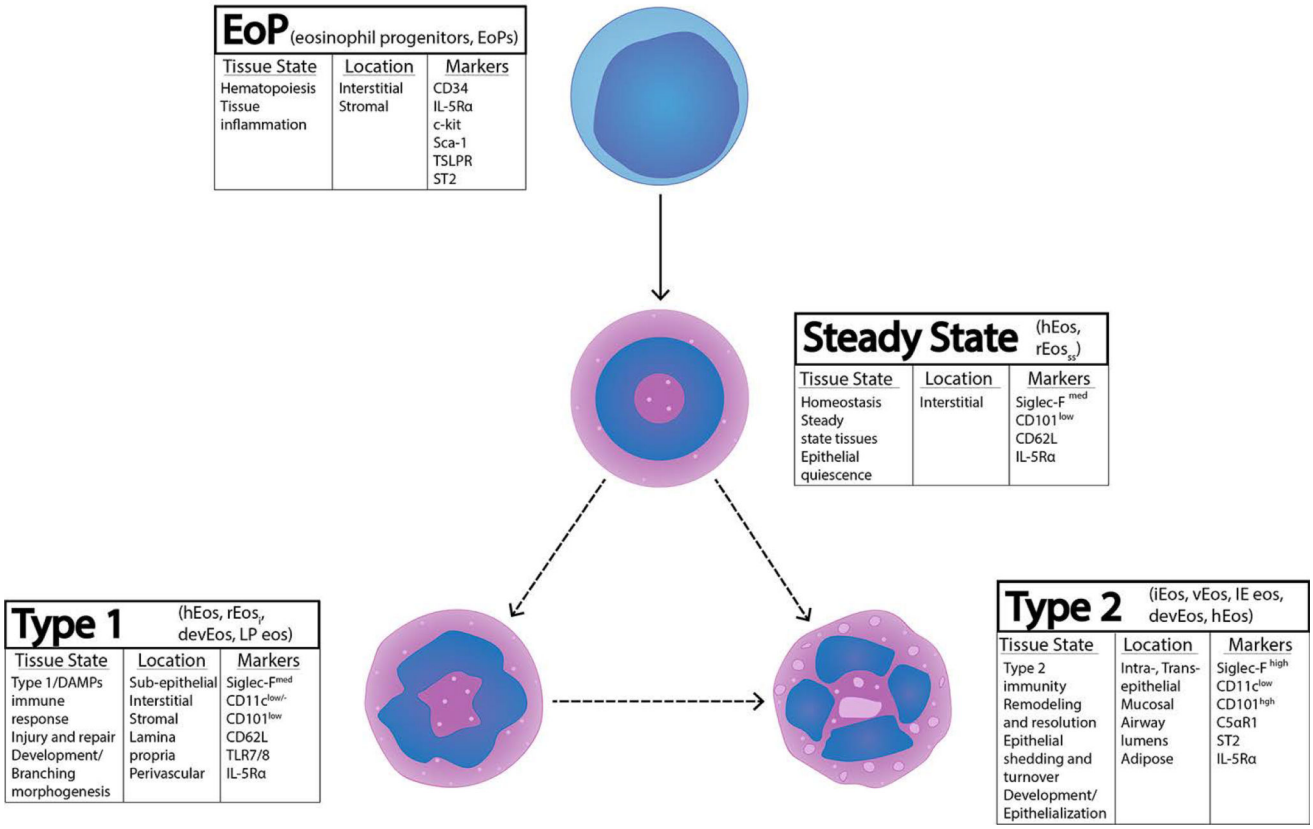

Figure 4.

Tissue-based classification of murine eosinophils. **EoP**: Eosinophil Progenitors - immature eosinophils or committed precursors undergoing *in situ* hematopoiesis. **Steady State**: true resident eosinophils in morphogenetically quiescent tissues, featuring non-segmented "donut-shape" nuclear morphology and eosin staining. **Type 1**: interstitial/stromal eosinophils found in transient morphogenetic contexts and during Type 1 immune activation, featuring segmented nuclear morphology but lacking vacuolarization. **Type 2**: eosinophils associated with the epithelium and Type 2 immune environment, characterized by highly segmented nuclei and the presence of vacuoles.