



Eosinophils in the Tumor Microenvironment

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Fabrizio Mattei, Sara Andreone,
Giancarlo Marone, Adriana Rosa Gambardella,
Stefania Loffredo, Gilda Varricchi,
and Giovanna Schiavoni

Abstract

Eosinophils are rare blood-circulating and tissue-infiltrating immune cells studied for decades in the context of allergic diseases and parasitic infections. Eosinophils can secrete a wide array of soluble mediators and effector molecules, with potential immunoregulatory activities in the tumor microenvironment (TME). These findings imply that these cells may play a role in cancer immunity. Despite these cells were known to infiltrate tumors since many years ago, their role in TME is gaining attention only recently. In this chapter, we will review the main biological functions of eosinophils that can be relevant within the TME. We will discuss how these cells may undergo phenotypic changes acquiring pro- or antitumorcidal properties

according to the surrounding stimuli. Moreover, we will analyze canonical (i.e., degranulation) and unconventional mechanisms (i.e., DNA traps, exosome secretion) employed by eosinophils in inflammatory contexts, which can be relevant for tumor immune responses. Finally, we will review the available preclinical models that could be employed for the study of the role in vivo of eosinophils in cancer.

Keywords

Angiogenesis · Cancer · Cationic proteins · CD8⁺ T cells · Cytotoxicity · Eosinophil · Exosomes · Extracellular Traps · Immune regulation · Lymphangiogenesis · Mast cell · Mouse models · Tumor Immunity · Tumor Microenvironment · Tumor prognostic value

F. Mattei · S. Andreone · A. R. Gambardella
G. Schiavoni (✉)
Department of Oncology and Molecular Medicine,
Istituto Superiore di Sanità, Rome, Italy
e-mail: giovanna.schiavoni@iss.it

G. Marone
Department of Public Health, University of Naples
Federico II, Naples, Italy

Azienda Ospedaliera Ospedali dei Colli - Monaldi
Hospital Pharmacy, Naples, Italy

S. Loffredo · G. Varricchi (✉)
Department of Translational Medical Sciences and
Center for Basic and Clinical Immunology Research
(CISI), University of Naples Federico II,
Naples, Italy

WAO Center of Excellence, Naples, Italy

Institute of Experimental Endocrinology and
Oncology “G. Salvatore” (IEOS), National Research
Council (CNR), Naples, Italy

List of Abbreviations

AEC	Absolute eosinophils count	PRR	Pattern recognition receptor
ANGPTs	Angiopoietins	PIGF	Placenta growth factor
ADCC	Antibody-dependent cell-mediated cytotoxicity	PDGF	Platelet-derived growth factor
Ag	Antigen	PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cells
APCs	Antigen-presenting cells	PD-1	Programmed cell death-1
<i>A. fumigatus</i>	<i>Aspergillus fumigatus</i>	PSF	Progression-free survival
BECs	Blood endothelial cells	ROS	Reactive oxygen species
BAL	Bronchoalveolar lavage	RSV	Respiratory syncytial virus
CAFs	Cancer-associated fibroblasts	Siglec-8	Sialic-binding immunoglobulin like lectin 8
CEL	Chronic eosinophilic leukemia	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
DCs	Dendritic cells	Th2	T helper 2
ECP	Eosinophil cationic protein	TSLP	Thymic stromal lymphopoietin
EDN	Eosinophil-derived neurotoxin	T _{REG}	T regulatory cell
EPX	Eosinophil peroxidase	TAM	Tumor-associated macrophages
<i>E. coli</i>	<i>Escherichia coli</i>	TATE	Tumor-associated tissue eosinophilia
EXO	Exosomes	TILs	Tumor-infiltrating lymphocytes
EV	Extracellular vesicles	TME	Tumor microenvironment
FGF	Fibroblast growth factor	TK	Tyrosine kinase
FPR-1	Formyl peptide receptor-1	VEGF	Vascular endothelial growth factor
HSC	Hematopoietic stem cell		
HMGB1	High Mobility Group Box 1		
HD	Hodgkin's disease		
HES	Hypereosinophilic syndrome		
ICIs	Immune checkpoint inhibitors		
ILC2	Innate lymphoid cells		
IFN	Interferon		
IL	Interleukin		
iNKT	Invariant natural killer T		
LIAR	Local immunity and/or remodeling/repair		
LECs	Lymphatic endothelial cells		
MBP	Major basic protein		
MCA	Methylcholanthrene		
mAb	Monoclonal antibody		
M-MDSC	Monocytic myeloid-derived suppressor cells		
MVB	Multivesicular bodies		
MDSC	Myeloid-derived suppressor cells		
NK	Natural killer () cells		
NO	Nitric oxide		
NOG	NOD/Shi- <i>scid</i> /IL-2R γ^{null}		
NSCLC	Non-small cell lung cancer		
OSCC	Oral squamous cell carcinoma		
OS	Overall survival		

1.1 Introduction

Eosinophils are rare blood circulating granulocytic cells representing 1–3% of total leukocyte population under physiological condition. Paul Ehrlich in 1879 first described blood eosinophils by their unique staining properties with acidic dyes, such as eosin and Luxol fast blue [1]. These cells originate and differentiate in the bone marrow in response to IL-5, together with IL-3 and GM-CSF, which support both maturation and survival of eosinophils [2]. In addition, IL-33 sustains eosinophilopoiesis at various levels, promoting survival, maturation, and functional activation [3]. During bone marrow development, IL-33 both expands eosinophil precursors expressing the IL-5R α and induces systemic IL-5 production, thus fueling the eosinophil maturation [4].

Upon response to certain inflammatory conditions (i.e., allergies, parasitic infections, and autoimmune diseases), eosinophils can rapidly

expand and can infiltrate inflamed tissues, where they play diverse roles in inflammatory responses. Eosinophils are well known to infiltrate the tumor microenvironment (TME), and this condition is referred to as tumor-associated tissue eosinophilia (TATE). The role of TATE in human cancers is still controversial [5, 6]. However, recent clinical observations in melanoma patients undergoing immunotherapy targeting the immune checkpoints CTLA-4 and PD-1 have unraveled a predictive role of eosinophil counts for therapeutic response [7]. These findings suggested that eosinophils might be regarded as possible prognostic/predictive biomarkers in cancer immunotherapy, thus repositioning this immune cell population at the forefront of cancer immunology research.

1.2 The Tumor Microenvironment: A Dynamic System with Multiple Interacting Players

The definition of tumor microenvironment (TME) originates from the dynamic interaction of the host immune system with the forming and growing tumor. This continuously evolving milieu is the result of the constant cross-talk between cancer cells and immune cells through the release of soluble factors that shape the phenotype of both cell types [8]. The TME is composed of a number of resident and nonresident cell types, as well as extracellular factors, and each cell component has a distinct role in this complex scenario [9]. When the TME is in its initial stage, resident tumor cells instruct the TME for the formation of blood vessels that allow the access of nutritive factors, cell-derived vesicles, and immune cells. Pericytes and endocytes, key cellular components of the blood vessel architecture, are considered resident cells in the TME [9] and play a relevant role in angiogenesis. In particular, a type-2 (Nestin⁺) subset of pericytes has been identified that promotes normal and tumoral angiogenesis [10]. Cancer-associated fibroblasts (CAFs), a heterogeneous subset of several cell

types, are resident cells that play an important role in tumorigenesis. These cells produce and release several mediators, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and cytokines, important to generate the 3D stromal architecture of blood vessels and of the TME itself [11].

The immune cells infiltrating the TME in solid cancers are heterogeneous, and their roles depend on the site, grade, and stage of malignancy. This is in part due to the fact that within the TME the patterns of soluble mediators (cytokines, chemokines, angiogenic, lymphangiogenic, and growth factors) and cellular receptors dynamically change and thus influence the homing and phenotype of immune cells [9].

Tumor infiltrating lymphocytes (TILs) are associated with antitumor activity, whose frequency often correlates with a favorable prognosis in cancer patients. In particular, CD8⁺ T cells are often present as infiltrating cells in solid cancers, where they can exert potent and selective cytotoxic action on tumor cells [12]. However, an important fraction of TILs is represented by regulatory CD4⁺ T (T_{REG}) lymphocytes with opposite effects on cancer progression. Indeed T_{REG} are endowed with potent pro-tumoral effects when infiltrating the TME and are considered a target for immunotherapeutic strategies [13]. Recently, a novel subset of tissue-resident memory CD69⁺CD103⁺ T cells (T_{RM}), either CD4⁺ or CD8⁺, has been reported to play a crucial role in preventing the development and spread of solid tumors and has been associated with favorable outcomes in cancer patients. T_{RM} cells may mediate tumor protection by promoting tumor-immune equilibrium through the secretion of cytokines and/or via CD103-enhanced tumor cell killing [14]. Natural killer (NK) cells, an innate immune subset with potent cytotoxic function, also contribute to tumor rejection [15].

Several dendritic cell (DC) subsets may be found in variable frequencies in the TME of various solid cancers, where they are deputed to tumor antigen (Ag) presentation and cross-presentation in lymphoid organs and in the TME itself [16]. Certain chemotherapeutic drugs pro-

mote the release of immunogenic signals from dying tumor cells, which are perceived by DC and promote a cascade of events that stimulate an anticancer immune response [17]. Among these signals, the ligand Annexin-a1 released by dying tumor cells was shown to bind formyl peptide receptor-1 (FPR-1), expressed by DC, acting as signal for the correct positioning of DC in proximity of dying cancer cells within TME. This Annexin-a1/FPR-1 axis enabled stable DC-corpse interactions, and subsequent engulfment and Ag cross-presentation by DC [18]. Ag cross-presentation for CD8⁺ T-cell cross-priming is mainly carried out by Batf3- and Irf8-dependent type I conventional DCs, a subset of DC expressing the markers CD103 and CD8 α [19]. CD8⁺ T-cell cross-priming is promoted by type I IFNs signaling on CD8 α DC and is required for antitumor immunity in vivo [20]. Type I IFNs act on CD8 α DC prolonging Ag retention after engulfment of tumor apoptotic cells leading to efficient CD8⁺ T-cell cross-priming [21]. In addition, DC can interact with innate and innate-like immune cells, including NK, invariant natural killer T (iNKT), and $\gamma\delta$ T cells, amplifying direct and indirect antitumoral responses through a mutual cross-talk [22]. On the other hand, some tolerogenic DC contribute to the generation of T_{REG} and engage in a cross-talk, thus favoring the establishment and maintenance of an immunosuppressive TME that inhibits antitumor immunity [23].

Myeloid cells represent a major fraction of infiltrating immune cells. Tumor-associated macrophages (TAM) play a major role in tumor progression. TAM are distinguished into two major subsets: classically activated M1 with antitumor functions and pro-inflammatory M2 that supports tumor progression [24]. The balance of frequencies in infiltrating M1 and M2 TAMs often dictates the tumor fate and is a prognostic factor for patients [25]. Myeloid-derived suppressor cells (MDSC) are immature-like myeloid cells capable of strong immunosuppressive activity. Based on their phenotype marker expression and morphology, these cells can be subdivided into two subgroups, monocytic MDSC (M-MDSC) and granulocytic or polymorphonuclear MDSC (PMN-MDSC) due to their morphological (but

not functional) resemblance with monocytes and granulocytes, respectively [25]. Both types of MDSC infiltrate the TME, where they act as potent suppressors of CD4⁺ and CD8⁺ T lymphocytes while favoring recruitment of T_{REG} cells. In addition, MDSC promote tumor cell stemness, angiogenesis, and metastasis [25]. Mast cells, [26], neutrophils [27], eosinophils [6], and basophils [28], historically recognized for their involvement in allergy and inflammation, are now being repositioned for the recently discovered role in cancer. The function and role of eosinophils within the TME will be covered in detail below.

1.3 General Properties of Eosinophils

For many years, eosinophils have been mostly appreciated for two aspects of immune response: the ability to fight parasites and their contribution to allergic inflammation [29, 30]. This is because eosinophils produce a wide array of toxic granule proteins and pro-inflammatory mediators that lead to tissue damage [31]. Indeed, eosinophils exert potent cytotoxic functions through the production and release of cationic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPX), and eosinophil-derived neurotoxin (EDN). Furthermore, eosinophils secrete a wide array of soluble mediators, including cytokines, chemokines, and angiogenic and lipid mediators, contributing to immune regulation, tissue remodeling, and many other processes [30].

Eosinophil degranulation can occur via different cellular mechanisms [32]. Eosinophils adherent to parasites have been shown to degranulate through classical exocytosis, a process involving granule fusion with the plasma membrane that creates a pore through which the total granule content is secreted into the target cell. In contrast, piecemeal degranulation enables the release “piece-by-piece” of specific granule-stored proteins, such as cytokines and chemokines, and is thought to be the main secretion mode during chronic inflammatory responses. As a third mode

of secretion, eosinophils may undergo cytolysis, a process involving extracellular release of intact granules with rupture of plasma membrane. Eosinophils may also undergo cytolytic cell death with extrusion of nuclear materials, such as histones and DNA, and extracellular expulsion of intact granules entrapped in DNA nets, named DNA traps [33, 34].

According to the *LIAR* hypothesis formulated by James Lee, eosinophils are homeostatic cells that regulate Local Immunity And/or Remodeling/Repair during both steady state conditions and disease, especially associated with tissue injury [35]. Hence, besides the destructive effects, eosinophils also participate in resolution of inflammation, tissue repair, remodeling, and homeostasis, through the release of a variety of pro-fibrotic (i.e., TGF- β), growth factors (i.e., FGF-2, NGF, and VEGF), and matrix metalloproteinases. In addition, eosinophils participate in the modulation of adaptive immune responses [36]. Eosinophils can induce the recruitment of Th2 cells [37] and T_{REG} cells [38] through the production of the chemokines CCL17 and CCL22. Moreover, eosinophil-derived CXCL9 and CXCL10 recruit Th1 cells [37, 39] and CD8⁺ T cells [40–42]. Eosinophil cationic proteins can have immunostimulatory activities; for example, EDN can both attract and induce the maturation of DC into a Th2-promoting phenotype [43]. Stimulation of eosinophils with CpG-ODN results in degranulation and induction of DC maturation in a cell contact independent manner via MBP [44]. Furthermore, EPX activates DC in vitro and in vivo, inducing mobilization to lymph nodes and Th2 priming [45]. Eosinophils play an active role in the induction and expansion of Th2 type of immune response, through the production of IL-4, IL-5, IL-13, and IL-25 participating in allergic reactions, parasitic infections, and autoimmune disorders [46]. Eosinophils can also produce, store, and secrete Th1-associated pro-inflammatory cytokines (i.e., IFN- γ , TNF- α and IL-12) and T_{REG}-associated mediators (i.e., IDO, IL-10, and TGF- β), thus demonstrating their versatile immunoregulatory role [46]. Following activation with cytokines, such as GM-CSF, IL-4, IL-5, or IFN- γ , eosino-

phils upregulate MHC class II and co-stimulatory molecules (CD80, CD86, and CD40) and can act as non-professional Ag presenting cells (APC) stimulating Ag-specific CD4⁺ T-cell proliferation and Th2 cytokine production in vitro and in vivo [47].

Eosinophils are equipped with a variety of surface receptors fundamental for their function and localization within inflamed tissues [30]. These include pattern recognition receptors (PRRs), such as TLR1–5, TLR7, TLR9, NOD1, NOD2, Dectin-1, and RAGE, that recognize specific molecular components associated with pathogens or danger signals and allow a rapid pro-inflammatory response to insults through production of cytokines, chemokines, and granule cationic proteins [48]. Eosinophils also express receptors for many cytokines (IL-2R, IL-3R, IL-4R, IL-5R α , IL-9R, IL-10R, IL-13R, IL-17R, IL-23R, IL-27R, IL-31R, IL-33/ST2, TSLPR, GM-CSFR, IFN γ R, TGF- β R), chemokines (CCR1, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CXCR2, CXCR3, CXCR4), formyl peptide receptors-1, -2, -3, and a variety of integrins and adhesion molecules (CD11a/CD18, CD11b/CD18, CD11c/CD18, CD49d/CD29, CD49f/CD29, ICAM-1), which drive eosinophil transmigration from the bloodstream to inflamed tissues [49, 50].

Eosinophils express various receptors for immunoglobulins, complements, proteases, and lipid mediators, such as leukotrienes and prostaglandins. Sialic-binding immunoglobulin-like lectin 8 (Siglec-8) and its ortholog murine Siglec-F are hallmark receptors for eosinophils that function as inducers of apoptosis associated with reactive oxygen species (ROS) production following antibody cross-linking [51, 52], especially when eosinophils are pre-activated with cytokines [53, 54]. Administration of Siglec-F mAb in vivo results in selective ablation of blood and tissue eosinophils in mice through induction of apoptosis [55]. EGF-like module containing mucin-like hormone receptor-1 (EMR1), the human ortholog of mouse F4/80, is a receptor highly specific to mature human eosinophils [56]. Targeting EMR1 with a specific mAb enhanced NK-mediated killing of human eosinophils

in vitro and induced eosinophil depletion in monkeys [57]. In addition, eosinophils express various adhesion molecules and integrins (i.e., CD49d/29, CD49f/29, CD11b/18, CD11a/18, CD11c/18, CD11d/18, and CD49d/ β 7) that are upregulated upon activation and mediate eosinophil migration and effector functions [58]. Overall, these features endow eosinophils with multiple roles as effectors and regulators of different immune responses.

1.4 Eosinophils in Allergic Diseases and Infections

Eosinophils play a prominent role in Th2-related pathologies, and tissue eosinophilia is associated with inflammation in respiratory allergies, atopic dermatitis, eosinophilic esophagitis, and gastroenteritis [30]. Allergic asthma is often associated with skewing of naïve Th cells toward Th2 phenotype and activation of eosinophils. In the latter condition, referred to as eosinophilic asthma [59], eosinophils are recruited in the airways by Th2 cytokines (i.e., IL-5) and chemokines (i.e., eotaxin-1/CCL11). Airway epithelial cells activated by different stimuli (e.g., allergens, super-allergens, viral and bacterial proteins, tobacco smoke, and so on) release the alarmins IL-25, IL-33, and TSLP that promote Th2 polarization with massive production of IL-4, IL-5, and IL-13 [60]. Recent evidence suggests that these alarmins stimulate type 2 innate lymphoid cells (ILC2), which also secrete IL-4, IL-5, and IL-13 and subsequently recruit eosinophils to the inflamed tissue [61].

Eosinophils are key players in airway inflammation contributing to the so-called T2 asthma pathogenesis by damaging the epithelium and orchestrating the immune response [62]. It is believed that the pathophysiologic effects of eosinophils in allergic inflammation are caused by the release of cationic proteins, ROS, lipid mediators, proteases, and pro-inflammatory cytokines. The mechanisms triggering eosinophil degranulation in inflamed tissues are not fully understood. However, recent evidences have shown that epithelial cell-derived alarmins may

play a role. In mouse models of allergic asthma, accumulation of eosinophils in the lung and ensuing allergic inflammation are strongly inhibited by blockade of IL-33/ST2 signaling pathway [63–66], while they are exacerbated by administration of recombinant IL-33 [67]. In patients, a rare IL-33 loss-of-function causes reduced number of eosinophils in blood and protects against asthma [68]. Furthermore, IL-33 is a potent activator of eosinophils in vitro, enhancing adhesion and promoting degranulation [69–71]. These data strongly suggest that IL-33 not only drives eosinophilia but also stimulates eosinophil effector functions in allergy. TSLP, another epithelial-derived alarmin involved in allergic inflammatory response [60], can promote degranulation and survival of eosinophils through STAT5 phosphorylation [72]. In mice, intradermal administrations of TSLP resulted in the induction of a systemic Th2-skewed inflammatory response, which was dependent on the presence of eosinophils [73]. A monoclonal antibody (mAb) targeting TSLP (tezepelumab) is currently under development for the treatment of different forms of severe type 2 asthma (i.e., eosinophilic and non-eosinophilic) [74, 75].

A canonical function of eosinophils is to provide protection against parasitic helminths. Eosinophils also participate in the host defense against other pathogens, such as bacteria, viruses, and fungi [49, 76]. The mechanisms accounting for the antiparasitic role of eosinophils in vitro include direct killing through the release of cytotoxic proteins (MBP, EPX, ECP, EDN) [77, 78]. Furthermore, eosinophils can present parasite-specific Ags to T cells in vivo, leading to the polarization of Th2 response and increase of Ag-specific IgM concentration [79]. However, studies in mouse models of helminth infection have yielded contrasting results, and the role of eosinophils in host response to parasites in vivo remains controversial [49]. In vitro, eosinophils adhere to the fungus *Alternaria alternata* by binding of the integrin CD11b to β -glucan, a component of the fungal cell wall, resulting in degranulation and release of the cationic proteins MBP and EDN [80]. In 1978, DeChatelet and coworkers first reported a bactericidal activity of

eosinophils, showing that these cells can phagocytize *Escherichia coli* or *Staphylococcus aureus* as efficiently as neutrophils via hydrogen peroxide production [81]. Furthermore, human eosinophils can release extracellular DNA traps in response to *Aspergillus fumigatus* in vitro, via a cytolytic mechanism that depends on the Syk tyrosine kinase pathway and CD11b [82]. Eosinophils exploit extracellular traps of mitochondrial DNA and granule proteins also to kill bacteria both in vitro and in vivo [34]. These catapult-like released traps protected from microbial sepsis in a model of intestinal inflammation, unravelling the importance of eosinophils for maintaining the intestinal barrier function after inflammation-associated epithelial cell damage.

Eosinophils play a role in host defense against single-stranded RNA viruses, such as respiratory syncytial virus (RSV), by exploiting the ribonuclease activity of the granule proteins EDN and ECP [83]. In vitro, degranulation of eosinophils following RSV-infected pulmonary epithelial cells is dependent on CD18-mediated cell contact [84]. In addition, binding of rhinovirus to eosinophils via ICAM-1 causes phenotypic activation of eosinophils promoting their ability to present viral Ags to Ag-specific T cells, causing T-cell proliferation and secretion of IFN- γ [85]. In vivo, eosinophils promote the clearance of RSV by stimulation of the TLR-7-MyD88 pathway, which triggers degranulation and expression of IRF-7, IFN- β , and iNOS [86]. Furthermore, infection of eosinophils with pulmonary viruses can result in the release of proinflammatory mediators, such as IL-6, IP-10, CCL2, and CCL3 [87]. The precise mechanisms by which eosinophils interact with viruses and contribute to host antiviral immunity remain to be clarified.

1.5 Repositioning Eosinophils in Cancer

The increase of eosinophils in cancer patients has been known for over a century. Pioneering studies in the 1980s described tumor-infiltrating eosinophils in human gastric cancers and suggested their good prognostic value for prolonged

survival [88, 89]. In addition, eosinophils were reported to exert cytotoxic function against breast cancer cells in vitro [89], and blood eosinophil counts inversely correlated with risk of recurrent disease in breast cancer patients [90]. In cancer patients undergoing immunotherapy with IL-2, eosinophils were expanded and acquired an activated phenotype, with increased degranulation, survival, and antitumor cytotoxicity [91–93]. Despite these early evidences supporting the presence of eosinophils in TME of many human cancers, the role of eosinophils in cancer has been largely overlooked for long time. Recent data indicate that these cells are potent immune effectors and regulators within the TME with potential prognostic/predictive role in human cancers.

1.5.1 Role of Eosinophils in Hematologic Tumors

The role of eosinophils in hematologic tumors is still unclear. Andersen and coworkers showed that the eosinophil counts below 0.16×10^9 /L can be associated with acute myeloid leukemia and myelodysplastic syndrome. By contrast, eosinophil counts above 0.16×10^9 /L were associated with myeloproliferative neoplasms [94]. Several studies have reported the association of peripheral blood and tissue eosinophilia with Hodgkin's disease (HD) [95]. Eosinophilia in peripheral blood is associated with a positive prognostic factor [96]. Eosinophils can be found in lymph nodes of HD patients, but their prognostic relevance remains controversial [95]. It is unclear why tissue eosinophilia can be found only in some subsets of HD patients. Tumor cells can produce different eosinophil-attracting molecules such as IL-5 and eotaxins [97, 98]. In nodular sclerosing Hodgkin's disease, tissue eosinophilia was shown to represent a poor prognostic indicator. In fact, binding of eosinophil-secreted CD30 ligand to CD30 on tumor cells was shown to trigger NF- κ B activation and consequent proliferation of tumor cells [99].

Verstovsek and coworkers highlighted the efficacy of alemtuzumab in the treatment of

patients affected by hypereosinophilic syndrome (HES) or chronic eosinophilic leukemia (CEL) refractory to standard therapies [100]. Alemtuzumab induced a relevant decline of the absolute eosinophilic count and the percentage of eosinophils in the peripheral blood in all 11 patients examined. This study demonstrated that alemtuzumab decreased blood eosinophils and improved patients with HES and CEL refractory to standard therapy. These case studies suggest, on one hand, that resistance to standard therapy may be in part due to the hypereosinophilia and, on the other hand, that treatment of these refractory patients with alternative therapies may be successful and is partly associated with a decrease of blood eosinophilia [100]. These data have been confirmed by Strati and coworkers, who reported that alemtuzumab is a useful treatment for CEL patients with hypereosinophilia [101].

1.5.2 Role of the Eosinophils in Solid Tumors

Several studies have shown an improved prognosis of patients with TATE or evidence of eosinophil degranulation in various types of solid tumors. Caruso and coworkers described TATE in human gastric adenocarcinoma, with tumor cells in close proximity to eosinophils displaying signs of autophagic cell death [102]. The same group described the presence of degranulating eosinophils in human advanced gastric carcinoma. In these ultrastructural studies, deposition of extracellular granules from apoptotic eosinophils either free in the tumor stroma [103] or within the cytoplasm of gastric carcinoma cells was observed [104].

An antitumoral role of eosinophils has been described in colon cancer, melanoma, lung cancer, and oral squamous cell carcinoma [6]. In melanoma mouse models, eosinophils play a clear antitumoral role both in restraining tumor growth and in preventing lung metastasis onset [40, 41, 105]. Lucarini et al. demonstrated the positive influence of eosinophils on CD8⁺ T cells for the local tumor, and their tumor cytotoxicity activity on lung metastasis [41]. Importantly,

eosinophil count in peripheral blood is correlated with a good prognosis in patients with metastatic melanoma undergoing immunotherapy with immune checkpoint inhibitors targeting CTLA-4 [106, 107] or PD-1 [108]. Furthermore, in a study involving 173 patients, it was shown that peripheral blood eosinophilia is a good prognostic marker correlating with prolonged survival in patients with metastatic melanoma independently from any treatment [109].

In mouse and human colorectal cancer, Munitz and colleagues elegantly demonstrated an antitumorigenic role of eosinophils during tumor development [110]. By analyzing human biopsies, they found an inverse correlation between tumor stage and intratumoral eosinophil counts. In *Apc^{min/+}* mice, which develop spontaneous intestinal adenomas, eosinophils were recruited into tumors during induction of inflammation-induced colorectal cancer and played an essential role in tumor rejection, independently of CD8⁺ T cells [110].

In non-small-cell lung cancer (NSCLC), a recent report by Tanizaki and colleagues showed an important correlation between peripheral blood eosinophil counts and patients' survival. They showed that an increase in absolute eosinophil count (AEC) (≥ 150 / μ l) is linked to a better progression-free survival (PSF) and overall survival (OS) in patients treated with nivolumab, an anti-PD-1 mAb. For this reason, they suggested that absolute eosinophil count can be a biomarker for this kind of treatment [111]. Dorta and colleagues reported an antitumor role of eosinophils in oral squamous cell carcinoma (OSCC). Indeed, they demonstrated that patients with a lower number of TATE have lower probability to survive [112].

By contrast, elevated eosinophils in the TME seem to be correlated with poor survival in cervical carcinoma patients [113]. This pro-tumoral activity of eosinophils may be related to the microenvironment of this type of cancer. In this regard, eosinophils may be polarized to a phenotype that promotes tumor growth and reduces tumor cell death [114]. The ensemble of these evidences suggests that the role of eosinophils in tumorigenesis is cancer dependent. Alternatively,

as discussed below, different subsets of eosinophils may exist that play divergent roles in tumorigenesis, depending on the tumor histotype.

1.6 Antitumoral Mechanisms of Eosinophils

Tumor-infiltrating eosinophils have the potential to control tumor progression, exerting direct and indirect antitumoral activities, through secretion of a variety of soluble mediators. Recruitment of eosinophils to the TME can be driven by several chemokines (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, and RANTES) that activate the CCR3 receptor highly expressed on eosinophils [6]. The alarmin IL-33, locally expressed by epithelial and tumor cells, can also promote eosinophil recruitment through stimulation of tumor-derived eosinophil-attracting chemokines [71]. IL-33, together with IL-5, may also prolong the life span of eosinophils at site of tumor growth [6].

Eosinophils accumulate early within tumor necrotic areas of experimental tumors, and this event is accompanied with degranulation and release of MBP and EPX [115, 116]. In fact, danger signals released by necrotic cells, particularly High Mobility Group Box 1 (HMGB1), can induce eosinophil migration, adhesion, survival, and degranulation with release of granule cationic proteins and ROS that promote oxidation and thus inactivation of necrotic material and tumor-promoting inflammation [116]. Moreover, eosinophil-derived MBP can inhibit the activity of heparanase, an endoglycosidase involved in remodeling the extracellular matrix that enhances tumor growth, angiogenesis, and the formation of metastasis [117].

1.6.1 Direct Antitumor Activity of Eosinophils

Eosinophils are equipped with granule proteins endowed with potent cytotoxic activity [118]. MBP can mediate tumor toxicity through disruption of membrane lipid bilayers [119]. ECP is

cytotoxic for Hodgkin lymphoma tumor cells [120], inhibits the proliferation of oral squamous cell carcinoma [121], and induces apoptosis of bronchial epithelial cells by caspase-8 activation [122]. Furthermore, EPX and EDN exhibit cytolytic activity against human colorectal carcinoma cell lines [123]. These findings support the hypothesis that eosinophil cationic proteins can exert direct antitumor activities. Vadas and coworkers reported that exposure of eosinophils to CSF or GM-CSF activates antibody-dependent cell-mediated cytotoxicity (ADCC) against EL-4 and BW thymoma cells, as well as P815 mastocytoma cells. Importantly, eosinophils needed direct contact with P815 cells to induce killing, suggesting a contact-dependent cytotoxicity [124]. Murine eosinophils also could induce apoptosis of A20 murine lymphoma cells through the release of granzyme B [125]. Furthermore, eosinophils activated by cross-linking of the 2B4/CD244 receptor exhibited tumoricidal activities against human B lymphoma cells [126]. In addition, human eosinophils can directly kill human colon carcinoma cells via release of granzyme A in a mechanism dependent on the integrin CD11a/CD18 and on IL-18 [123, 127]. These *in vitro* results suggest that tumor cytotoxic function of eosinophils require cell–cell contact through various adhesion molecules and integrins.

Whether eosinophils mediate tumor cytotoxicity *in vivo* remains to be demonstrated. Presence of degranulating eosinophils and of eosinophil-specific granules within tumor cytoplasm has been reported in human gastric cancer biopsies [103, 104]. In mouse melanoma models, immunotherapy with the “alarmin” IL-33 promotes the expansion and tumor infiltration of eosinophils, which play an essential role in antitumor responses mediated by IL-33 and prevents the onset of pulmonary metastasis. Terminal differentiation of bone marrow-derived eosinophils with IL-33 results in the generation of highly activated cells, compared to classical eosinophils differentiated with IL-5, revealed by increased aggregation in clusters (Fig. 1.1). IL-33-activated eosinophils highly resembled pulmonary eosinophils recruited by IL-33 *in vivo* and exhibited upregulation of granzyme B and potent tumor

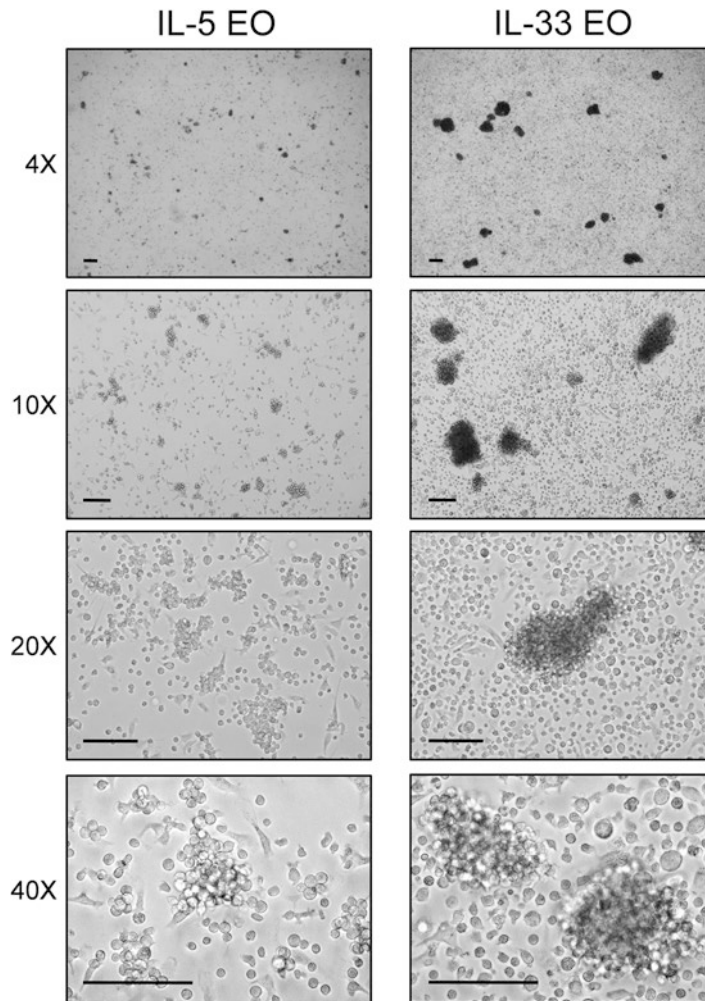


Fig. 1.1 Terminal differentiation of bone marrow-derived eosinophils with IL-33 yields highly activated cells. Bone marrow cells from tibiae and femurs of C57Bl/6 mice were cultured for 4 days in a medium containing 100 ng/ml SCF and 100 ng/ml FLT-3 L, followed by 10 ng/ml IL-5. From day 10, cells were supplemented every other day with either IL-5 or IL-33 (100 ng/ml) in order to generate IL-5 eosinophils (IL-5 EO) or IL-33

eosinophils (IL-33 EO), as described previously [36]. On day 16, when fully differentiated eosinophils were generated, microphotographs were obtained by EVOS-FL microscope. Images at the indicated magnifications show IL-33 EO aggregating in clusters, as opposed to IL-5 EO, indicative of highly activated phenotype. Scale bars, 100 μ m

cytotoxicity in vitro [6, 41]. Furthermore, IL-33-activated eosinophils are able to establish a large number of cell conjugates with different tumor cell lines (B16.F10 melanoma, MC38 colon carcinoma, TC-1 lung adenocarcinoma and MCA205 fibrosarcoma) leading to efficient tumor cell killing in vitro and in vivo [71]. IL-33 promoted the tumoricidal functions of eosinophils in a cell adhesion-dependent manner

through the integrin CD11b/CD18 and by inducing lytic granule convergence, with polarization of eosinophil effector proteins (ECP, EPX, and granzyme B) to the tumor-eosinophil immune synapse [71], in a similar mechanism operated by NK cells [128]. These observations demonstrate that IL-33 can potently stimulate eosinophil-dependent direct tumor cell killing by targeted degranulation, as schematized in Fig. 1.2.

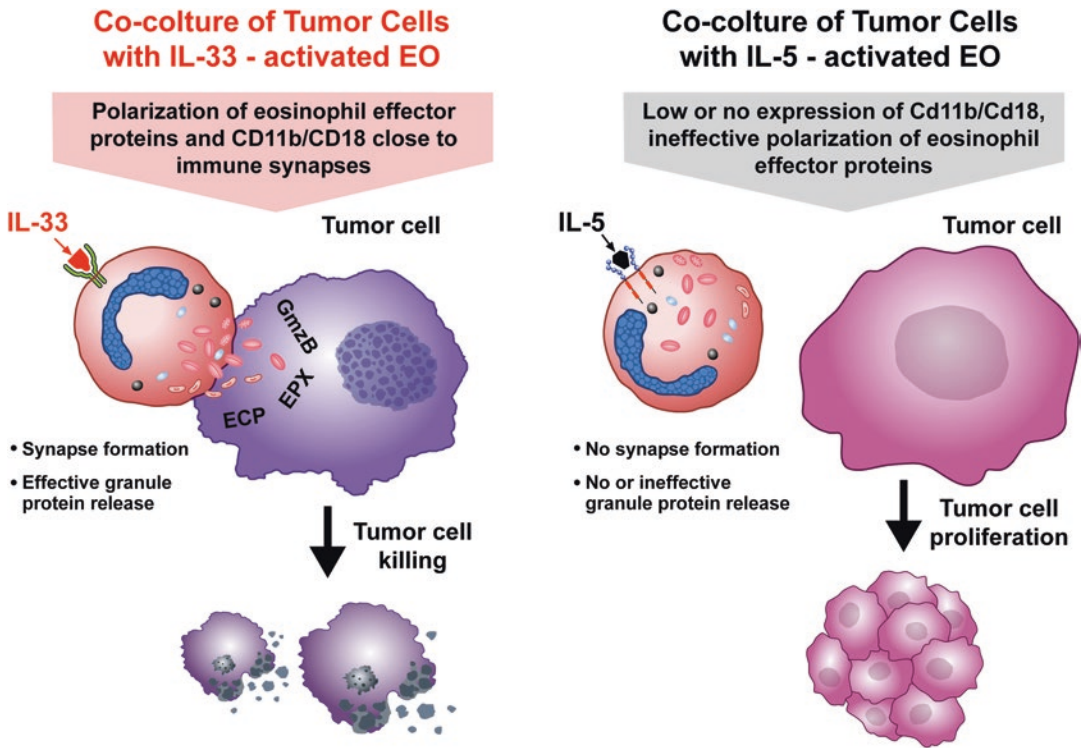


Fig. 1.2 IL-33 promotes the activation of CD11b/CD18 adhesion-dependent granule polarization in eosinophils within the immune synapse. Upon coculture with tumor cells, eosinophils (EO) activated with IL-33 through its specific receptor complex ST2/IL1RAP form stable EO-tumor cell conjugates. This event is mediated by CD11b/CD18-dependent adhesion and synapse-polarized

degranulation of eosinophil toxic proteins (EPX, ECP, granzyme B), resulting in efficient tumor cell killing. By contrast, activation of eosinophils with IL-5, through its receptor complex IL5RA/IL3RB subunit, fails to induce tumor cell adhesion and subsequent degranulation, thus sustaining tumor cell proliferation

1.6.2 Indirect Antitumoral Function of Eosinophils: Interaction with Other Immune Cells within the TME

Studies in preclinical models indicate that tumor-infiltrating eosinophils may affect indirectly tumor growth. In mouse models of melanoma, it has been shown that infiltrating eosinophils promote the recruitment of tumor-reactive CD8⁺ T cells through expression of the T-cell-attracting chemokines CCL5, CXCL9, and CXCL10 [40, 41]. Another indirect antitumor mechanism operated by eosinophils is their ability of influencing the tumor angiogenesis in TME. Human eosinophils can produce in vitro several proangiogenic molecules [129–132]. In vivo eosinophils induce

vessel normalization by increasing the expression of adhesion molecules, such as VCAM-1, and by polarizing TAM toward M1-like macrophages, which produce smaller amounts of proangiogenic factors, compared to M2 macrophages [40]. Finally, as discussed above, eosinophils may function as nonprofessional APC, although whether they do so within the TME remains to be demonstrated.

Both solid and hematologic tumors are associated with the accumulation of peritumoral and/or intratumoral mast cells, suggesting that these cells can help to promote and/or limit tumorigenesis [133]. Interestingly, human mast cells and eosinophils were both identified and named by Paul Ehrlich [134, 135]. These cells have distinct progenitors and differ morphologically, ultra-

structurally, immunologically, and biochemically. However, mast cells and eosinophils can form the “allergic effector unit” and can be found in proximity in TME of several tumors [135]. Therefore, it is likely that eosinophils have the capacity to modulate mast cell functions and vice versa. For example, ECP and MBP [136] and VEGFs released by activated eosinophils [137] can modulate mast cell functions. These bidirectional interactions between eosinophils and mast cells and vice versa might be relevant in TME.

1.7 Functional Plasticity of Eosinophils in Cancer

Eosinophils display the potential to interact with the tumor moiety. This feature stems from the ability of eosinophils to change their phenotype in response to stimuli present in the TME, such as cytokines, inducing variable responses. In addition, eosinophils have the capacity to release extracellular vesicles, which may shape the TME. For these reasons, eosinophils can be considered as cells endowed with a certain functional plasticity constantly remodeling the TME.

1.7.1 Role of Cytokines in Shaping Eosinophil Phenotype within the TME

It has been suggested that at least four subsets of murine eosinophils exist depending on their tissue localization, maturation, and type of immune response triggered [138]. The first subset is represented by eosinophil progenitors, which are immature eosinophils undergoing hematopoiesis in situ. They express the receptors for IL-5, IL-33, and TSLP, the latter two regulating eosinophil homing to inflamed tissues and activation. Steady-state or tissue-resident eosinophils, which were only characterized in the lung parenchyma of mice, are resting cells expressing intermediate levels of Siglec-F and with donut-shaped nucleus. A third subset (i.e., type 1 eosinophils) was described as interstitial/stromal cells in morphogenetic and type 1 immunity contexts. These

eosinophils display similar surface markers as steady-state eosinophils, such as Siglec-F^{int}, but have pluri-lobated nucleus without vacuolization. The fourth subset (i.e., type 2 eosinophils) is characterized in the epithelium in type 2 immunity contexts, such as allergic asthma and chronic colitis. These eosinophils exhibit pluri-lobated nucleus, vacuolized cytoplasm and high expression of Siglec-F. The relative roles of these subsets of eosinophils in cancer immunity are unknown. This is because these cell types have been characterized mainly morphologically and by the expression of some surface markers. Thus, gene expression profiles and single-cell RNA sequencing of tumor-infiltrating eosinophils in various settings may help to define these cells in relation to their functional (pro- or antitumorigenic) role.

Some studies have reported that cytokines may shape the phenotype of eosinophils, determining their polarization and tumor immune responses induced. Reichman and colleagues reported that in experimental colorectal cancer, intratumoral eosinophils exhibit an IFN- γ -related signature, which prevented the development of colorectal cancer in mice. Furthermore, activation of resting peritoneal eosinophils with IFN- γ potentiated their ability to kill colorectal cancer cells in vitro [110]. Similarly, activation of eosinophils with IFN- γ plus TNF- α induced upregulation of T-cell-attracting chemokines (CCL5, CXCL9, and CXCL10), IFN- γ , TNF- α , and NOS2. These activated eosinophils reduced tumor growth, through recruitment of tumor-reactive CD8 T cells, when adoptively transferred in melanoma-bearing mice [40]. These data suggest that IFN- γ may skew eosinophils toward a type 1 immunity-promoting phenotype.

IL-33 is another important cytokine that may affect eosinophil phenotype within the TME. In experimental tumors, IL-33 promotes antitumor immunity through expansion and activation of eosinophils in vivo [41, 105]. In melanoma-bearing mice, depletion of eosinophils by an anti-Siglec-F antibody injections abrogated the therapeutic efficacy of IL-33, indicating that eosinophils were indispensable for IL-33 antitumoral function [41]. Exposure to IL-33 in vivo

induced in tumor-infiltrating eosinophil gene transcripts, which differ in the primary tumor site and pulmonary metastasis. At the primary tumor site, IL-33-recruited eosinophils expressed T-cell-attracting chemokines (CCL5, CXCL9, and CXCL10), but not effector molecules. Conversely, at the pulmonary site, eosinophils expressed high levels of granzyme B and IFN- γ , but not T-cell-attracting chemokines. These findings suggested indirect or direct antitumor functions of eosinophils at the primary or pulmonary site, respectively. Furthermore, *in vitro* activation of eosinophils with IL-33 resulted in upregulation of effector molecules (i.e., granzyme B), Th-1 (i.e., IL-12, TNF- α), and Th-2 cytokines (i.e., IL-10, IL-13) [41, 71]. Thus, IL-33 may polarize eosinophils to a mixed type 1 and 2 immunity phenotype that promotes antitumoral function.

Several lines of evidence indicate that IL-5 may not support antitumor reactions in eosinophils. An early study showed that IL-5 gene-transfected tumors promote eosinophil recruitment but not antitumor immunity [139]. In a Meth-A fibrosarcoma model, intratumoral injection of OK-432 (a derivative of penicillin) and fibrinogen induces local production of IL-5 that recruits in the tumor tissue eosinophils which, however, did not play a relevant role in tumor regression [140]. In a different model, IL-5 could facilitate experimental lung metastasis of various cancer cells by creating an allergic inflammatory environment with CCL22-producing eosinophils that recruited T_{REG} cells [38]. By contrast, IL-5 plays a major role in driving the recruitment of eosinophils at primary and metastatic sites, promoting antitumor responses in models of hepatocellular carcinoma [141], methylcholanthrene-induced fibrosarcoma [98], and melanoma [142]. These apparently contrasting findings are compatible with the hypothesis that IL-5 can play distinct or even opposite roles in modulation of tumorigenesis. The role of IL-5 in shaping the phenotype of eosinophils in TME from different cancers needs to be addressed more extensively. Fig. 1.3 summarizes the possible role of subsets of eosinophils in the TME and their modulation by cytokines (i.e., IFN- γ , IL-5, IL-33).

1.7.2 Role of Extracellular Vesicles in the Biological Activity of Eosinophils within the TME

There is compelling evidence that eosinophils can release exosomes (EXO) and other extracellular vesicles (EV). EXO represent a small population of vesicles produced by any kind of cell and reflect the molecular signature (made up of lipids, nucleic acids, and proteins) of their producing cells. Through transfer of their bioactive molecules from the cell of origin to the target cell or tissue, EXO contribute to intercellular communication and represent an important diagnostic biomarker in pathological conditions [143].

Although intercellular communication appears to be the one of the most important functions of EV, they also have specific molecules related to their biogenesis. In fact, EXO have been defined by their size, density, and expression of specific biomarkers such as proteins involved in targeting and adhesion (tetraspanins, integrins, adhesion molecules), multivesicular bodies (MVB) biogenesis and secretion-associated proteins (ALIX, Rab GTPase), chaperone proteins, and others. The distinction between eosinophilic granules and EV becomes increasingly difficult, due to many shared molecules expressed, such as CD63 [144], and to the fact that granules can also be found intact extracellularly as membrane-bound, ligand-responsive structures [145]. Eosinophil EXO include/contain a series of cationic proteins (MBP, EPX, EDN, ECP), miRNA, mRNA, cytokines, chemokines, enzymes, and lipid mediators whose activities can mediate and autoregulate eosinophil biological functions. EXO-stored products are released from eosinophils through different mechanisms: classical exocytosis providing for exosome fusion with the plasma membrane and exosome embedding within target cell [146]. The release of eosinophil-derived EXO content in the receiving cell can condition the most important biological cell activities (transcription, translation, regulation by posttranscriptional or translational modifications), leading to drastic phenotypic variations in the receiving cell.

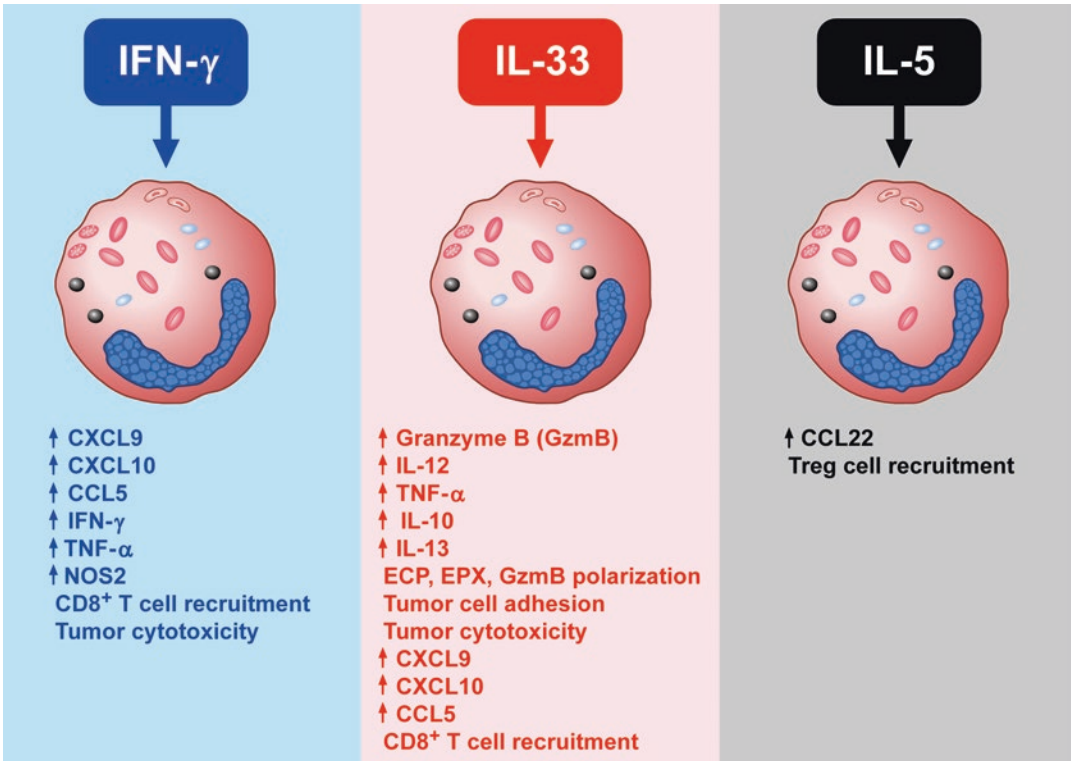


Fig. 1.3 Modulation of eosinophil phenotype by cytokines. Activation of eosinophils with IFN- γ results in the expression of T-cell-attracting chemokines (CXCL9, CXCL10, and CCL5), effector molecules (TNF- α , IFN- γ , and NOS2), promoting both CD8 T-cell recruitment and tumor cytotoxicity. Similarly, IL-33 triggering leads to upregulation of T-cell-attracting chemokines (CXCL9, CXCL10, and CCL5), effector molecules (granzyme B

and TNF- α), Th-1 cytokines (IL-12), and Th-2 cytokines (IL-10 and IL-13), as well as to granule protein polarization toward immune synapses. These traits favor eosinophil-mediated direct and indirect (CD8 T-cell-mediated) antitumor activities. In contrast, activation of eosinophils with IL-5 induce the expression of the chemokine CCL22 that recruits T_{REG} cells, which may promote tumor progression

Evidences suggest that eosinophil EXO, secreted into the extracellular microenvironment and delivered to different locations within the body, participate in multiple processes and pathologies, including asthma. Eosinophil EXO from asthmatic patients can influence the functions of structural lung cells, modifying several processes and changing the expression profile of various pro-inflammatory molecules [147]. Furthermore, eosinophil-derived EXO can increase nitric oxide (NO) and ROS production in eosinophils themselves, thus autoregulating eosinophil functions [148]. The role of eosinophil-derived EXO in cancer progression is unknown and deserves investigation. By using electron microscopy, Feng and coworkers demonstrated

that pericytes, like eosinophils, are equipped with internal vesicles that can be released outside of the cell [149]. Since pericytes represent an important component of vessels involved in the modulation of angiogenesis, it is conceivable that eosinophils and pericytes interact within the TME through the release of EV.

1.8 Regulatory Functions of Eosinophils: The Complex Role of the Angiogenesis

Angiogenesis and lymphangiogenesis are complex processes requiring a finely tuned balance between stimulatory and inhibitory signals [150–152]. The

formation of new blood and lymphatic vessels occurs vigorously during embryogenesis but is restricted in adults [150]. In adults, angiogenesis and lymphangiogenesis are limited to sites of chronic inflammation [64], tissue injury or remodeling [153], and cancer [154]. The association between angiogenesis/lymphangiogenesis and tumor growth was of great interest during the last decades for the implications of the nature of tumors and the possibility to inhibit cancer growth and the formation of metastasis by blocking angiogenesis/lymphangiogenesis [155]. The interest in angiogenesis/lymphangiogenesis increased during last years for several reasons. Chronic low-grade inflammation is an essential hallmark of cancer [8] and several immune cells can be involved, directly and indirectly, in the modulation of angiogenesis and lymphangiogenesis [6, 156–163]. The latter observation led to the recognition that the interactions between immune cells and the vascular system are involved in a multitude of cancers [164].

Angiogenesis is initiated by activation of vascular endothelial growth factor receptor 2 (VEGFR2), expressed on blood endothelial cells (BECs) by vascular endothelial growth factor-A (VEGF-A). Cancer cells are an important source of VEGF-A and other pro-angiogenic mediators [6, 165, 166]. Immune cells in TME increase VEGF-A availability during the angiogenic switch [159]. Angiogenesis and lymphangiogenesis require the participation of additional molecules, such as angiopoietins (ANGPTs) [167]. VEGF-A signaling through VEGFR2 is the major angiogenic pathway. VEGF-C and VEGF-D, mainly through the engagement of VEGFR3 on lymphatic endothelial cells (LECs), induce lymphangiogenesis in tumors and stimulate the formation of metastasis [168,169]. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF) [159]. VEGF-A signaling through VEGFR2 activates angiogenesis by inducing the survival, proliferation, sprouting, and migration of BECs. VEGF-A also increases endothelial permeability [160, 170] and induces inflammation [157, 163, 171]. There are several splicing isoforms of VEGF-A (121, 165, 189, and 206), which differ in their binding to matrix and to co-receptor.

VEGF-A₁₂₁ is freely diffusible, whereas VEGF-A₁₆₅, VEGF-A₁₈₉, and VEGF-A₂₀₆ bind to heparin and heparin proteoglycans on cellular surfaces and extracellular matrices [172].

VEGF-A is primarily known for its essential role in physiologic and pathologic angiogenesis [173] and also retains lymphangiogenic properties [174] by binding to VEGFR2/VEGFR3 heterodimer receptor [175]. VEGF-A modulates lymphangiogenesis also indirectly by recruiting immune cells (e.g., macrophages, mast cells) that produce VEGF-C and VEGF-D [161, 163]. PIGF, expressed in the placenta, heart, and lungs, has four isoforms (PIGF1–4) [176, 177]. VEGF-B is highly expressed in heart, skeletal muscles, and brown fat in adults and has two major isoforms in humans: VEGF-B₁₆₇ binds to heparin proteoglycans, whereas VEGF-B₁₈₆ does not bind heparin and is more soluble [178]. PIGF and VEGF-B bind with high affinity to VEGFR1 whose tyrosine kinase (TK) activity is weak and downstream signaling poorly understood [179]. VEGFR1 is expressed on BECs, some immune cells, and pericytes, and its TK activity is required for cell migration toward VEGFs or PIGF [157, 171, 180]. VEGF-B and PIGF are angiogenic in certain pathophysiological settings [181]. VEGF-B can modulate coronary vessel growth and cardiac hypertrophy and lipid metabolism [177, 182].

The VEGF-C/VEGFR3 signaling pathway is the main pathway implicated in lymphangiogenesis [183]. VEGF-C is crucial for the survival, proliferation, and migration of LECs [184]. VEGF-D also binds VEGFR3 to promote lymphangiogenesis [168].

Angiopoietins (ANGPTs) also play an important role in modulating angiogenesis and lymphangiogenesis. In humans, the ANGPT/Tie system consists of two cell-surface TK receptors (TIE1 and TIE2) and two ligands ANGPT1 and ANGPT2. TIE2, primarily expressed on BECs, binds both ANGPTs, whereas TIE1 is an orphan receptor that can modulate ANGPT1, expressed by perivascular cells (i.e., pericytes), and sustains BEC survival. By contrast, ANGPT2, secreted by BECs, acts autocrinally and paracrinely as TIE2 ligand to promote angiogenesis and lymphangiogenesis [185]. Several chemokines, produced by

immune and nonimmune cells, also play a role in the modulation of angiogenesis and antiangiogenesis [158].

Human eosinophils produce several angiogenic factors such as VEGF-A [129, 137], fibroblast growth factors (FGF-2) [40, 130], CXCL8/IL-8 [131], and osteopontin [132]. Human eosinophils also produce MMP9 [186–188]. Eosinophils have been detected in metastatic lymph nodes of cancer patients, but the production of lymphangiogenic factors by these cells should be further addressed.

1.9 Mouse Models to Investigate the Role of Eosinophils in Cancer

Several mouse models have been developed for the study of the functional role of eosinophils. Most of these experimental models have been employed mainly in the study of respiratory diseases, such as asthma and eosinophilic esophagitis. These models can be transgenic, genetically engineered, target-specific, and humanized.

1.9.1 Transgenic Mouse Models

A relevant transgenic mouse model used to assess phenotypic features of eosinophils in the host is represented by PHIL transgenic mouse model of study. PHIL mice were first described by Lee and colleagues as a transgenic line of mice with a complete ablation of eosinophils and the contemporary presence of a fully functional hematopoietic compartment [189]. These mice have been generated by replacing the eosinophil peroxidase (EPX) with the Diphtheria toxin A chain (DT) and by exploiting the cytotoxic property of DT. When host eosinophils undergo maturation or activation of the transcription factors devoted to the expression initiation of EPX in PHIL transgenic mice, the promoter transcribes the replaced DT sequence, thus selectively ablating eosinophils [189]. These mice have allowed to establish the contribution of eosinophils to the resolution of inflammatory responses in experi-

mental pulmonary allergies [190], experimental colitis [191], and to pathology and protection against parasites [78].

An alternative mouse model for studying eosinophil functions *in vivo* is the Δ dblGATA1 transgenic mice. Deletion of a high-affinity GATA site in the GATA-1 promoter results in a complete ablation of the eosinophil lineage without affecting the development of the other GATA-1-dependent lineages, such as erythroid, megakaryocytic, and mast cell [192]. These mice have been used to demonstrate a protective role for eosinophils in a methylcholanthrene (MCA)-induced fibrosarcoma tumor mouse model [98]. However, besides eosinophil deficiency, Δ dblGATA1 mice were subsequently reported to display numerical and functional aberrancy in basophils [193], thus raising concerns on their specificity for eosinophil-specific studies.

IL-5 transgenic mice display an overrepresented eosinophil compartment due to the insurgescence of eosinophilia in the host. These hypereosinophilic mice display abnormally high presence of eosinophils in bone marrow, spleen, and peritoneal exudate, compared to controls. Simson and coworkers proposed a role for eosinophils in tumor immunosurveillance by using the IL-5 transgenic mice with elevated levels of circulating eosinophils [98]. Similarly, Kataoka and coworkers exploited these IL-5 transgenic mice to demonstrate an antitumor activity of eosinophils in hepatocellular carcinoma [141].

1.9.2 Target-Specific *In Vivo* Models

Depletion mechanisms to generate selected target-specific mouse models have been developed to produce *in vivo* eosinophil-targeted models of study. Repeated systemic injections of an anti-Siglec-F polyclonal antibody that functionally inhibits the activity of Siglec-F protein and selectively induces apoptosis in eosinophils result in eosinophil ablation in mice [55]. Recently, this Siglec-F-based functional depletion of eosinophils in mice has been employed to study the role of eosinophils in cancer. By using this approach, three independent groups demon-

strated the essential role of eosinophils in antitumor response in mouse models of melanoma and other cancers [40, 41, 105].

1.9.3 Genetically Engineered *In Vivo* Models

Initially, genetically modified mouse models with impaired eosinophil development and/or function, although not eosinophil-specific, were described. These include mice deficient for the eosinophilic cytokine IL-5 [194] or its receptor [195], which are characterized by the absence of eosinophilia upon Th2 cell-inducing stimuli. Furthermore, mice with a double deletion of CCL11 and CCL24 genes are characterized by a severe diminished eosinophil recruitment in response to allergic stimuli [196]. These models were largely employed in allergy and respiratory disease research.

Subsequently, mice deficient for eosinophil-specific granule proteins MBP and EPX were described. MBP-1^{-/-} mice were generated by truncating the MBP-1 gene, thus producing a dysfunctional protein containing the exons 1 and 2 but lacking the exons 2, 3, and 4. This model is characterized by a reorganization of eosinophil secondary granule structures and by a marked reduction of the eosinophil numbers in lung parenchyma and bronchoalveolar lavage [197]. Similarly, EPX^{-/-} mice were generated by a targeted disruption of the EPX gene, in which the normal EPX gene was replaced with a dysfunctional EPX sequence lacking the exons 7, 8, and 9. EPX^{-/-} mice displayed an altered structure of eosinophil secondary granules and a remarkable reduction of eosinophils in lung BAL [198]. Interestingly, a recent report described the generation of a double knockout mouse model for both MBP and EPX (MBP^{-/-} EPX^{-/-} mice). These mice are featured with eosinophil deficiencies similar to those observed in animals deficient of EPX or MBP only, but represent an advance in the implementation of an *in vivo* model to investigate eosinophil pathophysiology [199]. These models are largely utilized for research in allergy, inflammation, and

respiratory diseases and could be successfully employed in anticancer research [200].

1.9.4 Humanized Mouse Models

The development of humanized mouse models best recapitulates the pathology of human diseases and thus represents a major goal to understand the role of eosinophils in human cancer. Recently, a novel IL-3/GM-CSF/IL-5 Tg NOD/Shi-scid-IL2r γ null (NOG) model, a mouse strain in which human eosinophil differentiation is induced from HSC, was reported [201]. In this mouse strain, the authors established a human asthmatic inflammation model by intratracheal administration of human IL-33. This enabled to study the Th2 responses specifically in a human context, including the eosinophil-dependent responses in asthma. By using humanized NSG mice adoptively transferred with human CD34⁺ hematopoietic stem cells, Arnold and collaborators showed that following infection with gastrointestinal bacteria, eosinophils are recruited to the tissue where they reduce inflammation by suppressing Th1 immune responses [202]. Thus, humanized mouse models represent a valid opportunity to investigate specifically the roles of eosinophils in different human cancers.

1.10 Concluding Remarks and Outstanding Questions

There is compelling evidence that eosinophils are potent effector and immunoregulatory cells in TME of experimental and human cancer [49, 203]. Several studies have reported that eosinophilia can be associated with a favorable prognosis in a variety of solid and hematologic tumors [6]. By contrast, a limited number of studies indicate a protumorigenic role of eosinophils [6, 114]. The potentially dual role (i.e., protumorigenic and antitumorigenic) of eosinophils raises several fundamental questions. First, there is the possibility that the role of eosinophils and their mediator is cancer-specific (e.g., influenced by different TMEs). Alternatively, different sub-

sets of eosinophils and/or different eosinophil-derived mediators can play distinct or even opposite roles in tumorigenesis. There is already evidence, at least in mice, of the existence of different subsets representing different stages of maturation of eosinophils [202, 204, 205]. Recent fate mapping experiments demonstrate that macrophages [206] and mast cells [207, 208] form a highly heterogeneous population of immune cells, similar to T cells [209]. Future studies should address the possible roles of plasticity/hypothetical subtypes of eosinophils by single-cell RNA-seq, together with analyses of encoded proteins.

Studies of eosinophils are usually performed on cells isolated from peripheral blood where O₂ and nutrients are abundant and pH neutral. By contrast, eosinophils in TME are embedded in a hostile metabolic setting characterized by hypoxia, accumulation of lactate, potassium and adenosine, and low pH [210–214]. Thus, the biochemical and functional characteristics of peritumoral and intratumoral eosinophils likely differ from those of peripheral blood eosinophils.

Experimental models have started to provide evidence that eosinophils and their mediators can play a protective role by inhibiting tumor growth and the formation of metastasis in different cancers [40, 41]. Several mouse models have been characterized for the evaluation of the pathophysiological roles of eosinophils. Different groups have used target-specific mouse models to demonstrate the antitumorigenic role of eosinophils [40, 41, 105]. Humanized mouse models best recapitulate human disease and will represent a useful tool to evaluate the role of eosinophils and their mediators in different human cancers.

Immunotherapy with mAbs targeting immune checkpoint inhibitors (ICIs) (e.g., CTLA-4, PD-1/PD-L1 network) has revolutionized the therapies of an increasing number of solid and hematologic tumors [215, 216]. Unfortunately, these therapies are effective only in a percentage of patients, and there is urgent need of biomarkers predictive for ICI-based immunotherapy [217]. There is some evidence that baseline peripheral blood eosinophils represent a useful biomarker for prognosis of melanoma [109] and

NSCLC [111]. Future studies should evaluate the predictive value of different subsets of peripheral blood eosinophils (e.g., low and high density) in response to ICIs in different cancers.

A deeper insight into the immunological and molecular mechanisms regulating the link between tumor-infiltrating eosinophils and tumor cells could lead to the identification of new prognostic/predictive biomarkers, as well as a wider view of cancer immunotherapy, in an even more personalized therapeutic approach.

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