

HARNESSING EOSINOPHILS AS A POTENTIAL TUMORICIDAL
IMMUNOTHERAPY

A Thesis

by

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ABSTRACT

Eosinophils have numerous functions for defense against pathogens, despite being only 1-5% of total circulating blood leukocytes. Most well known for their actions contributing to asthma and allergic disease, eosinophils are thought to have homeostatic roles in the thymus, uterus, mammary glands, and gut. Furthermore, recent studies have also indicated a role for eosinophils as sentinels of tumorigenesis (2-6). Our studies show that eosinophils can elicit tumoricidal activity in a dose dependent manner against a variety of cancer cells. We also attempted to optimize the cytotoxicity of eosinophils by activating different receptors. We were able to verify different methods to stimulate eosinophil activation, including incubation with cytokines, especially granulocyte macrophage, colony stimulating factor (GM-CSF), and crosslinking the Fc α R. Eosinophils do have tumoricidal activity, but further research is needed to both understand this interaction and to optimize the eosinophil's tumoricidal properties.

DEDICATION

This work is dedicated to my family for giving me the emotional support needed to finish both this thesis and medical school. Special thanks to my husband, for his patience and consideration in the time needed for the completion of this work and for talking me out of every anxiety attack that occurred.

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CHAPTER I

INTRODUCTION*

Eosinophils have a wide repertoire of functions for defense against pathogens, despite being only 1-5% of total circulating blood leukocytes. Eosinophil differentiation and survival is predominantly regulated by interleukin-5 (IL-5), and eosinophil migration is largely in response to the chemokine eotaxin-1. Eosinophils are also considered important for the pathogenesis of asthma and allergic diseases. Thus, inhibiting eosinophil proliferation and activity by targeting IL-5 and its receptor subunit IL-5R α is a common therapeutic goal for these conditions [1].

Interestingly, eosinophils are found in basal levels in the thymus, uterus, mammary glands, and gut and are thought to have beneficial tissue homeostasis. Furthermore, recent studies have also indicated a role for eosinophils as sentinels of tumorigenesis. Firstly, tumor associated eosinophilia has been widely described with an improved prognosis in colon, breast, colorectal, nasopharyngeal, oral, gastric, and head and neck cancers [2-7]. More recently, murine studies have indicated that eotaxin is necessary to clear melanomas and that eotaxin deficient mice have lower tumor clearance. Furthermore, IL-5 transgenic mice have decreased tumor metastasis. A tumoricidal role for eosinophils against a human colorectal cancer cell line has been suggested, in vitro, and was cell contact-dependent and required the adhesion molecule CD11a/CD18 [8-10].

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We tested the hypothesis that eosinophils have the potential for tumoricidal activity against a broad spectrum of tumors, and thus have the potential to be harnessed as a novel tumoricidal immunotherapy. We did this in two parts. First, by investigating if eosinophils could attenuate tumor cell viability, and second, by investigating the mechanism of eosinophil activation and release of cytotoxic molecules in hopes to amplify the eosinophil's tumoricidal effect.

The Eosinophil

Eosinophils are innate immune effector cells best known for providing host defense against parasites, as well as playing a role in the pathogenesis of allergic diseases such as asthma, and in hypereosinophilic syndromes [1,11-13]. In the past decade, additional physiologic roles for eosinophils have emerged, which include coordination of tissue remodeling events, orchestration of homeostatic functions, and regulation of innate and adaptive immunity [1,11-13]. Typically, eosinophils are found in low numbers in the blood (1-4% of total peripheral blood leukocytes; less than 500/cu mm), and under homeostatic conditions are also found within mucosal tissues, as well as primary and secondary lymphoid organs [14]. Eosinophils can be rapidly generated from bone marrow progenitors and recruited to sites of inflammation. The cytokine, IL-5, is essential for the differentiation and survival of eosinophils from hematopoietic progenitors [15]. Eosinophils and their progenitors express the IL-5R which is composed of a ligand specific alpha chain (IL-5R α) and the common beta receptor (β c). The β c chain is shared by interleukin-3 receptor (IL-3R) and granulocyte macrophage-colony stimulating factor receptor (GM-CSFR) and combines with their respective α chains

[16]. Chemotactic molecules are necessary for eosinophil recruitment and migration. Eotaxin-1 is an eosinophil specific chemokine and is the most potent chemokine for eosinophils [17,18]. Other less selective chemokines include RANTES, eotaxin-2, eotaxin-3, monocyte chemoattractant protein (MCP) 2 and MCP-3 which also utilize the eotaxin-1 receptor (CCR3) [17,18].

Central to eosinophil effector functions is the capacity of these cells to immediately release their tissue-destructive cytoplasmic granules upon activation by various stimuli. Eosinophil granule secretion leads to the release of preformed pro-inflammatory mediators such as cytokines, chemokines, lipid and neuro-mediators, reactive oxygen species, growth factors, and cationic proteins [1,11-14]. Eosinophils are characterized by the presence of specific granules that contain four classic cationic proteins: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophilic cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) [1,11-14]. The collective destructive power of these cytotoxic proteins provides efficacy against infectious organisms, accounts for the bystander damage to host tissue during eosinophilic inflammation, and makes them potentially attractive candidates for use as tumoricidals. Eosinophils mediate airway remodeling via profibrotic cytokines, such as transforming growth factor beta (TGF β), and eosinophils are the largest producers of TGF β in the airway [19,20]. TGF β contributes to airway remodeling by detaching airway epithelial cells and increasing deposition of extracellular matrix proteins which causes fibrosis via matrix metalloproteinases (MMP) and IL-6 [19,20].

Eosinophils in Host Defense

Eosinophils are central to host immunity against parasites [21-24]. In addition, eosinophils can effectively participate in immunity to bacterial and viral infections via ligation of pattern recognition receptors by damage associated molecular pattern molecules (DAMPs) and pathogen associated molecular pattern molecules (PAMPs) to pattern recognition receptors [22,24]. Eosinophils are most effective against helminth parasites. When parasites infiltrate host tissues, a Th2 response is elicited which increases the generation of eosinophils under the influence of IL-5 [21,23]. Eosinophils are recruited to the site of infection by eotaxin-1. Once in contact with the parasite, the eosinophil degranulates to release reactive oxygen species and cytotoxic molecules such as EDN, EPO and MBP [23]. Eosinophils will also secrete lipid bodies which contain a variety of eicosanoids that are necessary (along with mast cells) for the smooth muscle modulation that occurs in peristalsis designed to expel parasites [23]. In contrast, eosinophils promote parasite infection by aiding in the establishment of *Trichinella spiralis*, while also limiting parasite load through the prevention of larval spread while still acting to eradicate the adult form[25,26].

Eosinophils in Allergic Inflammation and Eosinophilic Syndromes

Eosinophils have a firmly established role in allergic inflammatory responses. In humans and mice, one of the hallmarks of asthma is eosinophilic infiltration of the bronchial mucosa and submucosa, and the number of airway eosinophils is directly associated with disease severity in asthmatic patients [27,28]. A role for eosinophils in the pathogenesis of asthma was experimentally supported using eosinophil-deficient

mice, which had markedly diminished allergen-induced airway inflammation and markedly diminished bronchial hyper-reactivity [29,30]. Similar results were seen with IL-5 deficient mice, implicating the IL-5/eosinophil axis in allergic inflammation [31]. This axis was likewise implicated in humans by the presence of increased IL-5 in bronchoalveolar lavage fluid and bronchial biopsies of patients with allergic asthma [32-35].

Perhaps where eosinophilic pathology is most blatant is in hypereosinophilic syndromes (HES), which encompass a variety of disorders whose commonality is chronic elevation of blood eosinophil counts [36-38]. HES can cause complications such as cardiomyopathy, hepatosplenomegaly, neuropathy, skin lesions, and pulmonary disease. These symptoms can be fatal if untreated, and are secondary to the tissue damage caused by the eosinophils' toxic mediators. Subtypes of HES are being delineated, and include those with the PGDF-FIPL1 fusion gene, those secondary to increased IL-5 production, as well as idiopathic causes [36-38].

Other diseases characterized by eosinophilia include eosinophilic esophagitis (EE) and eosinophilic gastroenteritis (EG), referring to excess eosinophil infiltration of the esophagus and the stomach or intestines, respectively [39-41]. Experimentally, eotaxin deficient mice had attenuated EE and IL-5 deficient mice had complete ablation of EE [42]. EG can occur in all parts of the gastrointestinal tract, and like EE, is usually caused by allergic responses. Churg Strauss disease is also characterized by hypereosinophilia, and nasal polyposis is characterized by increased IL-5 production and infiltration of eosinophils in the polypoid tissue [43-45].

Duality of Eosinophil Physiology

The descriptions above demonstrate that eosinophils can be harmful or beneficial. There are two prevailing paradigms to explain this duality: either (1) the participation of eosinophils in allergic inflammation are part of a common physiologic Th2 immune response to environmental insults at the host-environment interface, or (2) eosinophils in allergic inflammation are a physiologically unintended or misdirected pathologic response that stems from the host's use of the Th2 anti-parasite immune response pathway [46]. In support of the first option, the LIAR hypothesis specifically emphasized that the role of eosinophils is to provide "Local Immunity and Remodeling Repair," explaining that the basal levels of eosinophils in the tissues are responsible for homeostatic remodeling [46,47]. Hence, the allergic response would be an over exuberant, intended response to potentially harmful environmental antigens. By extension, Th2 immune responses may have also specifically evolved against non-infectious noxious agents or toxins as a mechanism to promote behavioral change (like the avoidance venomous stinging or biting animals). Whether Th2 immune responses evolved independently against parasites and non-infectious noxious agents, or are based on a shared mechanism continues to be debated [46,47].

IL-5 and the IL-5 Receptor Complex

Eosinophils are critically dependent on IL-5 for their differentiation, activation, prolonged survival, increased adhesion to vascular endothelial cells, and augmentation of cytotoxic activity [1,11-13]. IL-5 is a glycoprotein homodimer that is produced by Th2 cells, as well as by natural killer (NK) cells, mast cells, basophils and eosinophils

[15]. Activated eosinophils produce IL-5 in an autocrine fashion to prolong their survival, and some evidence suggests that IL-5 is necessary for eosinophil migration along with chemokines like eotaxin-1 and RANTES [48].

IL-5 binds to a heteromeric receptor composed of a 65kD, high affinity, ligand-specific IL-5R α and a homodimeric 130kD β c, which is common to the GM-CSFR and the IL-3R [16]. Structurally the IL-5 homodimer is composed of two four alpha helix motifs A-D and A'-D' that are arranged in an up-up-down-down antiparallel configuration connected by loops [16,49]. The homodimer conformation interdigitates the A, B, and C helices from one molecule and the D' helix from the other molecule, thereby yielding a molecule with a pair of four alpha helical bundle motifs with a C2-axis of symmetry [50]. Although this structure provides the homodimer with two potential binding domains for IL-5R α and two for β c, only one IL-5R α has been shown to directly bind to IL-5 [50]. The structural interactions of IL-5R α -bound IL-5 to β c have yet to be solved, but are predicted to be limited to one β c. The IL-5 binding domain for IL-5R α lies within the 1st and 3rd antiparallel loops, while the β c binding domain is anchored by the glutamate-13 residue (Glu-13) of IL-5 [50,51]. A recent crystallography study showed that steric hindrance is responsible for only one IL-5R α being bound by the IL-5 homodimer [52]. It is predicted that IL-5 and the IL-5R complex forms in the same way that the GM-CSF and the GM-CSFR forms a dodecamer complex, with two IL-5/IL-5R α complexes binding to one β c, followed by further aggregation of these ligand/receptor complexes, which enables engagement of adjacent

β_c , thereby facilitating transphosphorylation of JAK2 and signal transduction via STAT5 (Figure 1) [53].

The IL-5R α extracellular region consists of 3 fibronectin type III domains (D1, D2, D3). D2 binds IL-5's M2 region using the D2 $\beta_1\beta_2$ loop while also binding at the hinge site between D2 and D3. It is D1 which is thought to be imperative for IL-5 binding, and dependent on Ile-161[54]. After IL-5 binds to IL-5R α via disulfide bonds, a conformational change occurs to allow IL-5 interaction with the β_c . Both β_c and IL-5R α are constitutively associated with JAK kinases, and are responsible for signal transduction [55]. The main signaling pathways involved are the JAK/STAT, Ras/MAPK, p38/NF κ B, and the phosphoinositide 3-kinase (PI3K) pathways. These signaling pathways direct the transcription of various genes involved in eosinophil differentiation, activation, and survival [55].

Antagonism of IL-5 and the IL-5 Receptor complex

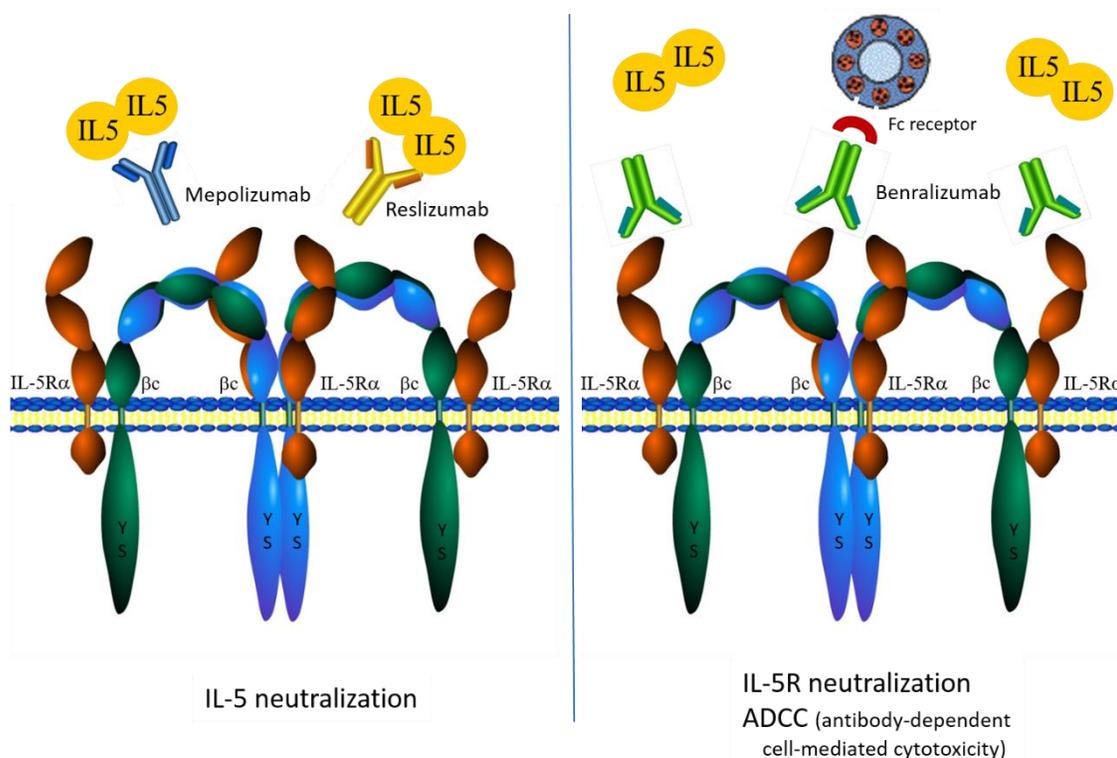


Figure 1. Therapeutic Strategies for Antagonizing the IL-5/IL-5R Axis.

In an inactivated state, the IL-5 receptor consists of two single IL-R chains (*dark orange chains*) and a c dimer (*blue and green chains*). Two approaches have been used to inhibit IL-5-induced signaling in eosinophils: (1) neutralization of IL-5 by humanized mAbs, mepolizumab and reslizumab (*left panel*); and (2) neutralization of the IL-5 receptor alpha chain (IL-5R) to block IL-5 binding and mediate ADCC lysis, benralizumab (*right panel*). The first blocks the formation of a signaling competent IL-5R complex (*left panel*). The second binds the cell surface IL-5R to prevent IL-5 binding altogether. However, this approach also leads to antibody-dependent cellular cytotoxicity (ADCC) caused by Fc receptor binding on NK cells to the anti-IL-5R mAb on eosinophils (*right panel*)

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Strategies for Antagonizing Eosinophils

As eosinophils play a contributing role to allergic inflammation, asthma, and hypereosinophilic syndromes, eosinophil depletion has been a tantalizing target for treatment of these conditions. Since IL-5 is a specific mediator of eosinophil differentiation and survival, IL-5 and its receptor have evolved as drug targets. Alternative strategies for antagonizing eosinophilic inflammation include targeting eotaxin, eosinophil adhesion molecules, or eosinophil signaling pathways. However, the greatest success has resided in targeting IL-5 or IL-5R α .

Targeting IL-5

Early on it was determined in murine models that IL-5 neutralizing antibodies were effective down regulators of eosinophilic inflammation, with similar favorable outcomes as those with IL-5- deficient or IL-5R α -deficient mice [56]. In developing anti-IL-5 neutralizing monoclonal antibodies (mAb), the crucial epitopes are within the β c binding domain and the IL-5R α binding domain. Targeting the β c binding domain on IL-5 would still allow IL-5 to bind to IL-5R α on the surface of eosinophils, but signaling could not occur since engagement of the β c would be blocked. Conversely, targeting the IL-5R α binding domain on IL-5 would block IL-5 binding to the eosinophil. Targeting either of these IL-5 domains would be predicted to be equally efficacious. Currently there are only two IL-5 neutralizing mAb in human use, mepolizumab and reslizumab. Both of these mAb have been humanized, bind to epitopes within the IL-5R α binding domain (Table 1), and bind to IL-5 with similar affinity, 4.2pM and 20pM, respectively [47-60]. Mepolizumab is an IgG1 κ antibody, while Reslizumab is an IgG4 κ antibody,

and hence exhibit differences in their Fc biologic activity with IgG4κ hveing poorer binding ffinity [58-60]. Whether their isotype differences will be clinically important is not clearly defined. Of note, there are no commercially developed mAb that bind the IL-5 βc binding domain, although this remains a viable target.

Target sites	Drug	Other names	Isotype	Mechanism	Human uses
IL-5 binding domain for IL-5Rα	Mepolizumab (GSK)	SB-240563	IgG1k	Neutralizing antibody	(Phase 2) Asthma
					(Phase 2) Adult eosinophilic esophagitis
		Bosatria			(Phase 2) Pediatric eosinophilic esophagitis
					(Phase 2) Churg Strauss syndrome
					(Phase 2) Nasal polyposis
					(Phase 3) Hypereosinophilic syndromes
					(Phase 3) Pediatric eosinophilic esophagitis
					(Phase 3) Rhinovirus induced asthma
			(Phase 3) COPD		
		Reslizumab (Cephalon)	SCH55700	IgG4k	Neutralizing antibody
		Cinquil	(Phase 2) Hypereosinophilic syndromes		
			(Phase 3) Pediatric eosinophilic esophagitis		
			(Phase 2) Loiasis		
IL-5 binding domain for βc	-----		-----	-----	-----
IL-5R α	Benralizumab (MedImmune)	MEDI-563	IgG1k	ADCC, competitive inhibition	(Phase 2) Asthma (Phase 2) COPD
IL-5R βc	TPI ASM8 (Pharmaxis)		-----	Antisense gene target	(Phase 2) Asthma
Eotaxin					

Table 1. Targeting IL-5 and the IL-5R.

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Mepolizumab

The initial mepolizumab trial targeted asthma patients. In this study, adult males with asthma received a single infusion of mepolizumab, and while peripheral blood eosinophil levels were reduced, there was no effect on clinical signs and symptoms [60,61]. Subsequent studies selected for patients with eosinophilic asthma and subgroups that were insufficiently controlled with corticosteroids. In prednisone- dependent asthmatics, an infusion of 750mg mepolizumab was administered once a month for five months. Patients receiving the intervention had decreased blood and sputum eosinophils and improved asthma control as judged by decreased asthma exacerbations and lower requirements for prednisone [62]. In another study in adults with corticosteroid-refractory asthma, twelve monthly doses of mepolizumab resulted in fewer exacerbations and patients improved their asthma quality of life score [63]. These trials also saw a subgroup improvement in patients with nasal polyposis. In an independent study on adults with severe nasal polyposis, patients who received two monthly infusions of 750mg mepolizumab had a significant reduction in blood ECP and soluble IL-5R α , and nasal IL-5R α , IL-6, and IL-1 β , which correlated with polyp improvement based on total polyp score (TPS) [64]. Mepolizumab was approved by the FDA in 2015 for use in patients with severe asthma attacks refractory to traditional treatment. Mepolizumab, named Nucala, will be given as subcutaneous injections every month in conjunction with other asthma medications [65].

Mepolizumab has been used against a variety of eosinophil-mediated diseases, and studies have shown remarkable clearance of blood, lung and bone marrow

eosinophils. Among the HES, mepolizumab trials have focused on FIP1L1–PDGFRA negative patients, since the FIP1L1–PDGFRA fusion gene promotes eosinophilia independent of IL-5 and is treated with the kinase inhibitor imatinib [66]. In patients requiring corticosteroid treatment for HES, 750mg of mepolizumab was administered intravenously every 4 weeks for 36 weeks. Of the patients who received mepolizumab, 84% lowered their prednisone dosage to below 10mg/day as compared to 43% of the placebo group which achieved this end point. The intervention group also had lower blood eosinophil numbers (95% less than 600/ μ L), and the placebo group had a shorter time to treatment failure. Overall, hypereosinophilia was better controlled in the intervention group [66]. To determine if mepolizumab was equally effective for the lymphocytic and non-lymphocytic subsets of HES patients, 750mg mepolizumab was administered every four weeks. This study showed that corticosteroid use could be reduced to a similar extent, but blood eosinophil numbers were not as attenuated in lymphocytic HES as they were in patients with non-lymphocytic HES [67].

When used to treat eosinophilic esophagitis, patients who were dysphagic (among other symptoms) received 10mg/kg mepolizumab (up to 750 mg) every 4 weeks for 3 total treatments. All patients had improved clinical outcomes related to decreased dysphagia, blood eosinophil levels were decreased 6-fold, and three of the four patients had decreased esophageal epithelial hyperplasia [68]. In a study that looked more closely at the molecular modulations, Straumann demonstrated that the improvement in dysphagia was likely due to reduction in tenascin C and TGF β 1 in the esophagus, although this study showed only mild clinical improvements [69]. To determine if

mepolizumab could be safely and effectively used in children, three monthly infusions of 0.55, 2.5, or 10 mg/kg mepolizumab were administered [70]. In children that had fewer than 20 eosinophils per high power field, there was an improvement in esophageal erythema, friability and furrows or vertical lines.

Mepolizumab has also been used successfully for patients with Churg Strauss disease [71]. In a case report of a 28 year old female, monthly infusions of 750mg mepolizumab reduced eosinophils to normal levels resolved the patient's asthma, and improved lung parenchyma by chest radiographs [72]. In a clinical trial of patients with Churg Strauss disease and marked eosinophilia, four monthly infusions of 750mg mepolizumab resulted in a 64% reduction of corticosteroid use at 12 weeks, and a 61% decrease at 24 weeks. Eosinophilia was also reduced, but upon cessation of the study exacerbations recurred [73].

Mepolizumab was unsuccessful in the treatment of atopic dermatitis [73,74]. In two studies by Oldhoff, mepolizumab did not improve patient prognosis as judged by physician global assessment (PGA), scoring atopic dermatitis SCORAD, and thymus and activation-regulated chemokine (TARC) scores and by atopy patch test. In these studies blood eosinophilia was reduced, but tissue eosinophilia was not [73,74].

Reslizumab

In a reslizumab pilot study, 1mg/kg reslizumab was administered intravenously once to patients with severe persistent asthma that was not controlled by corticosteroids [75]. Eosinophils were significantly reduced by about 50% after 2 days and slowly reestablished to about 18% 30 days after reslizumab intervention [75]. However, the

only noticeable improvement was increased forced expiratory volume (FEV) at the 24 hour post-treatment time point which was not sustained. In a later study of patients with poorly controlled asthma and sputum eosinophilia, the intervention group received monthly intravenous infusions of reslizumab. Results indicated that while all patients had attenuated eosinophil numbers, only the nasal polyposis subgroup showed increased lung performance based on an Asthma Control Questionnaire (ACQ) which indicates that reslizumab may be an important therapeutic for certain disease subgroups[76].

In a limited study for HES, a single infusion of reslizumab (1mg/kg) was administered to four adults with HES inadequately controlled by corticosteroids [77]. Three patients had significant reduction of eosinophilia, and two also had improved clinical symptoms. After cessation of treatment, eosinophil levels rebounded and exacerbations occurred. The fourth patient had no reduction in eosinophilia, with self-limited exacerbations [77].

Reslizumab treatment has also been used for pediatric eosinophilic esophagitis [78]. Patients received 1, 2 or 3mg/kg reslizumab infusions monthly for four months. While all groups had a reduction of eosinophils, complete clearing of the esophagus did not occur and esophagitis improvement did not correlate with eosinophil reduction [78].

This study reported minimal adverse outcomes, the most common being cough, headache, congestion and respiratory tract infection. Reslizumab is presently in further clinical trials for the aforementioned diseases to better elucidate the specificity of treatment, along with a clinical trial to evaluate its use in patients with loiasis, in an effort to limit host tissue damage associated with the loiasis-induced hypereosinophilia [79].

Targeting the IL-5 Receptor

There are two commercial therapeutics that target the IL-5R: benralizumab and TPI ASM8 [80-82]. Benralizumab is an IgG1 κ mAb specific for IL-5R α [80,81]. This drug has been developed to bind to the first fibronectin domain on IL-5R α which attenuates eosinophil number by competitively inhibiting binding of IL-5 to the IL-5R, as well as by antibody-dependent cell-mediated cytotoxicity (ADCC) via Fc γ RIIIa expressed by NK cells, macrophages and neutrophils (see Figure 1) [54,80,81]. The eosinophil lowering capability is effective up to 56 days after administration. Benralizumab has been shown to be effective in clinical trials with asthma patients in whom it reduced eosinophil numbers in a dose dependent manner, as well as reducing ECP levels. Benralizumab is also currently in phase II trials for the treatment of chronic obstructive pulmonary disease (COPD) [83].

TPI ASM8 is an antisense oligonucleotide that targets both the β c and eotaxin-1 [82]. While β c is an attractive target in that it would potentially inhibit the three Th2 cytokines, IL-5, IL-3, and GM-CSF, chronic treatment might result in pulmonary-alveolar proteinosis due to inadequate GM-CSF signaling [84,85]. Likewise, targeting β c would also target much broader subpopulations of leukocytes.

TPI ASM8 has been tested with 4 day and 14 day treatments [82]. These short-term treatment regimens and the short half-life of TPI ASM8 may mitigate potential chronic effects that could arise. In one study, it was determined that the half-life for the cocktail was less than 7 hours and that the drug did not accumulate overtime [82]. In another study, mild asthmatics were antigen challenged and then inhaled TPI ASM8

with increasing doses for 4 days (twice daily for the first three days and then once on the fourth day after challenge). After 7 hours there was a 60% reduction in sputum eosinophils and a 68% reduction after 24 hours. Likewise, ECP levels were reduced after 3 days with attenuation of both early and late asthma response [82].

Hansen et al. have described an anti- β_c mAb that antagonizes signaling in vitro [52]. The study showed that their anti- β_c mAb inhibited GM-CSF dependent colony formation by bone marrow cells from patients with chronic myelomonocytic leukemia, and hence might be a future therapeutic for such patients [52].

CHAPTER II

EOSINOPHILS AND TUMOR CELLS*

Cancer therapy is a double-edged sword whose goal is often described as killing the tumor before you kill the patient. This is because traditional cancer therapies- chemotherapy and radiation- target both healthy and compromised cells. Immunotherapy has been a burgeoning area of cancer research due to the innate ability of immune cells to act as sentinels to both external and internal threats to the body. Along with expanding the available anti-cancer drugs and decreasing mortality, by bringing cancer cells to the attention of immune cells, the hope is that cancer cells can be specifically targeted, thus sparing the healthy cells of the body, which would improve patients' quality of life.

Targets of Cancer Immunotherapy

Cancer is the result in a disturbance of cell cycle regulation. Cancer cells' success depends on numerous factors: the ability to stimulate their own growth, their resistance of inhibitory signals and to cell death, the potential to replicate indefinitely, angiogenesis, and invasion. Tumor suppressor genes (TSG) respond to aberrant cell proteins and thus shut down production of aberrant cells. This is why mutations in checkpoint regulating proteins- from tumor suppressor genes, like p53, are common in many cancer types [87].

Additionally, cancers have a worse prognosis with high endogenous reactive oxygen species (ROS) levels and with antioxidants suppression. Yet this tolerance can

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be overcome with excess ROS production, as in radiotherapy. Cancer cells have varying tolerance to ROS, and thus varying sensitivities to targets that utilize ROS. Cancer stem cells are a subpopulation that have low ROS sensitivity and high antioxidant activity that aids in resistance to cancer therapies [88, 89, 90].

Other checkpoint inhibitors include ipilimumab, an anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) mAb, as well as pembrolizumab and nivolumab, anti-programmed cell death protein 1 (PD1) mAb [91]. CTLA-4 targets a molecule expressed by Tregs that has a key role in dampening cytotoxic T cell activation [90]. Ipilimumab has been approved for treating unresectable or metastatic melanoma. PD1 is involved in dampening immune responses and binds to PDL1 and PDL2 which are expressed by tumor cells. Both the receptor and ligand were targeted in clinical trials and shown to be effective in non-small cell lung cancer (NSCLC) and melanoma. Nivolumab has been approved for treatment of unresectable melanoma [93].

Vaccines have also been used to treat cancers. For the latter, the most successful would be the human papilloma virus (HPV) vaccine, marketed under the name Gardasil. As a vaccine, it has no function in treating active cervical cancer but is a measure to prevent dysplasia, similar to the hepatitis vaccines. More interesting is the development of therapeutic vaccines such as those against prostate cancer and pancreatic cancer that have been shown to prolong survival [88,94,95].

Adoptive cell therapy involves removing the patient's own cells and enhancing them to better target tumor cells before returning them to the patient. Sipuleucel is FDA approved and uses dendritic cells to target antigen prostatic acid phosphatase-a specific

prostate cancer antigen, similarly T-cell therapies main goal has been to target expansion of CD8+ T cells and T cell activation by increasing tumor associated antigen immunogenicity [88].

Monoclonal antibodies are widely used to treat cancers. Some are cancer specific like trastuzumab which targets the HER2/Neu receptor and can only be used against HER2/Neu positive breast cancer, while others are more general like bevacizumab which inhibits angiogenesis via VEGF inhibition, and is used for multiple types of cancer [88, 96].

Eosinophils as a Tumoricide

Although targeting CD8+ cytotoxic T cells and Th1 cytokines has been much studied in cancer immunotherapy, tumor associated tissue eosinophilia (TATE) is widely recognized and poorly understood. [2-7, 97-100]. The induction of a Th1 immune response (by M1 macrophages) is most commonly associated with increased tumor control and better prognosis of disease, while the Th2 immune response and its related molecules may exacerbate tumor growth and decrease tumor control [101,102]. Nonetheless TATE in some cancers has been associated with an improved prognosis [2-7]. Hence, there is no agreement as to whether or not the presence of eosinophils is beneficial or detrimental to patient outcomes.

As reported by van Driel, TATE has a poor prognosis in cervical cancer, while Ishibashi reported that TATE in esophageal squamous cell carcinoma has no correlation with prognosis [97,98]. To investigate the potential effect of eosinophils on carcinomas, Wong compared the effect of IL-5 neutralizing antibody treatment to placebo on

chemically induced squamous cell tumors in hamsters [99]. Eosinophil levels were decreased and the tumor burden was lower in anti-IL-5 treated hamsters. Thus, in this model, eosinophils appeared to be contributing to tumor pathogenesis. Eosinophils are involved in tissue remodeling and they produce VEGF as well as induce endothelial cell production of VEGF [103, 104]. Furthermore, eosinophils produce pro-angiogenic cytokines: GM-CSF, FGF and TGF α , as well as matrix degrading enzymes in the form of MMP, all of which are associated with remodeling [105-108]. Together, this data suggest that eosinophils could be used by tumor cells to promote their survival and expansion.

Conversely, patient survival or time to recurrence was improved in patients with TATE in some, colon, breast, colorectal, nasopharyngeal, oral, gastric, and head and neck cancers [2-7], and metastasis was less frequent in colon cancer and head and neck cancer [3,4]. These studies have suggested that eosinophils could be used as a prognostic indicator such that patients with TATE could receive less aggressive interventions.

Few studies have been performed to elucidate the connection between eosinophils and tumor prognosis. In mice, B16 OVA melanoma tumor clearance was dependent on eotaxin and degranulating eosinophils [9]. Additionally, IL-5 transgenic (Tg) mice, which overexpress IL-5 and have hypereosinophilia, had decreased tumor burden after fibrosarcoma induction, and eotaxin deficient mice had greater fibrosarcoma burden. Eosinophil encapsulation of the tumor was prolonged in IL-5Tg mice which may account for arrest of tumor growth [109]. Cormier furthers this

observation in mice by demonstrating that eosinophil accumulation occurs early in subcutaneously injected melanoma tumors and localizes specifically to necrotic tumor areas and encapsulated areas [10]. A study performed in vitro on human derived colon cancer cells demonstrated that eosinophils can kill tumor cells in a cell contact-dependent mechanism requiring the adhesion molecules CD11a and CD18, and that key molecules involved in the cytotoxic effects were TNF, ECP and Granzyme A [8].

Materials and Methods

Cancer Cell Culture

Human derived cancer cell lines were maintained in culture based on ATTC guidelines and in accordance to our IRB guidelines, incubated at 37°C and fed every 48 hours. Prostate cancer cells, PC3, and erythroleukemic cells, TFI, were grown in Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS). NSCLC cells, A549, were grown in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, and RKO, colon cancer cells were grown in Kaighn's modified Ham's medium (F-12K) with 10% FBS. Cells were treated with trypsin when splitting or harvesting cells.

Eosinophil Isolation and Culture

De-identified human blood Leukopaks containing 50-70mL of blood were obtained from Gulf Coast Regional Blood Center and diluted 1:5 with HBSS, layered on Ficoll-Paque, and centrifuged 700 x g for 30 min at 18°C. The pellet was harvested and red blood cells were lysed and washed away and the remaining granulocytes were loaded

into a Robosep with mAb negative selection for eosinophils, using an eosinophil purification mAb cocktail (StemCell Technologies).

Purity was measured by flow cytometer. Dual staining was performed with CD16 and CD66b. The eosinophil population is CD66b⁺ and CD16⁻, while a double positive population would be indicative of neutrophils. Purity was confirmed by light microscopy with a Wright-Giemsa stain. Cells of less than 90% eosinophil purity were not used for experimentation. Eosinophils were either used directly after isolation or incubated at 37°F overnight in RPMI media with 10% FBS at a concentration of 1 million cells /mL. Unless otherwise noted, cells incubated overnight were also treated with 10ng/mL GM-CSF and IL-5, while some of the experiments also used other cytokines, discussed below.

Co-culture

Fresh or overnight incubated eosinophils were cultured with cancer cells at varying ratios. Cancer cells were trypsinized, washed, and plated overnight from the stock culture at a concentration of 1 million cancer cells per mL media. Conditions varied between experiments and they will be discussed in detail in the results section.

Measuring Cytotoxicity

Cytotoxicity was measured by tumor cell death via tumor cell count, and by determining viability of tumor cells cultured with and without eosinophils. A cellometer was used to count tumor cells and size was used to distinguish tumor cells from eosinophils which are easily differentiated by cellometer or flow cytometer. To measure viability, flow cytometry analysis was performed after staining with both Annexin V,

which is indicative of apoptosis, and propidium iodide (PI), which intercalates into nucleic acids and is considered to be a definitive marker of cell death.

Results

PC3 Cell Count Reduction Directly Correlates to Eosinophil Concentration

In our preliminary experiments PC3 cells were co-cultured for 4 hours with eosinophils measuring effector:target ratios of 0.1:1 to 20:1. The data showed a reduction in tumor cell count that directly correlated to increasing concentrations of eosinophils (Figure 2A, 2B). When the data was standardized the dose dependency varied between experiments (Figure 2C). For inter-assay standardization we used the following formula:

$$\frac{\text{\# viable tumor cells after co-culture}}{\text{\# tumor cells without eosinophils}} \times 100$$

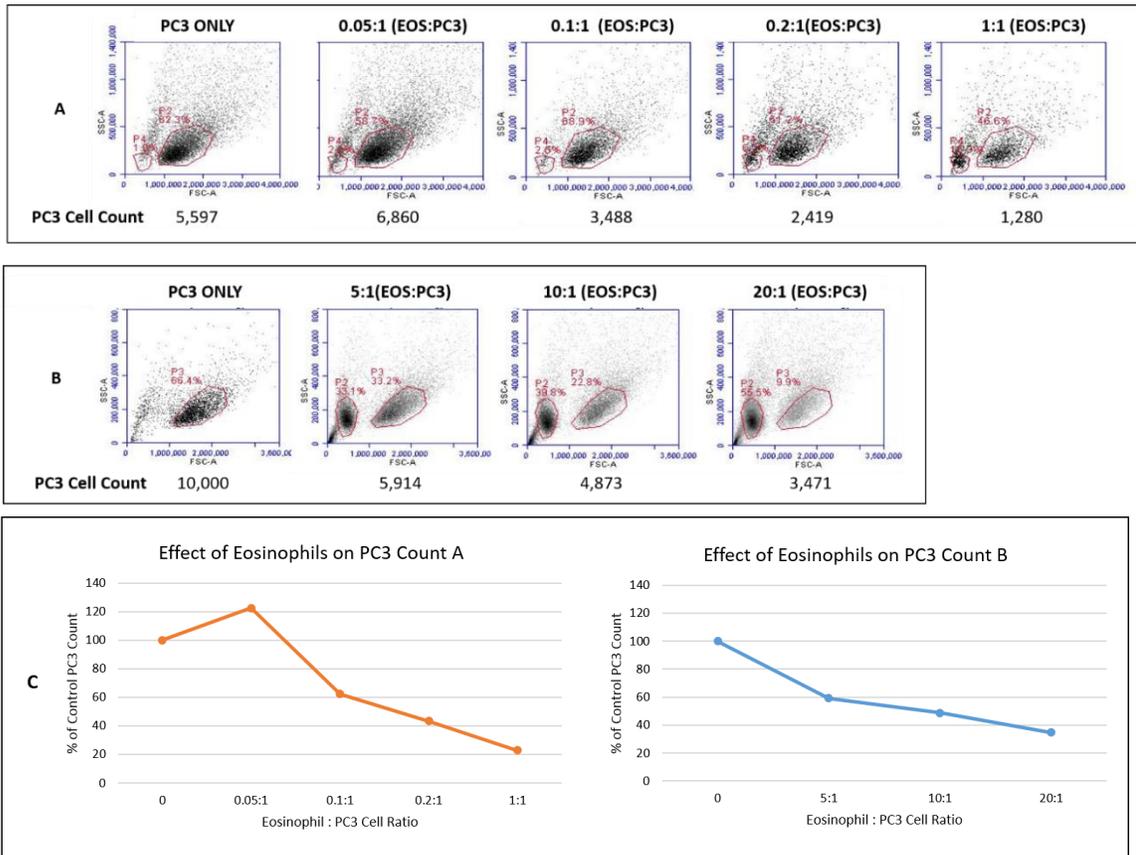


Figure 2. Eosinophils Decrease PC3 Count in a Concentration-dependent Manner. PC3 cancer cells were plated on 12-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs in the indicated ratios. Viability was assessed via Annexin V and PI. The cells were gated based on the pure PC3 (CTL) scatterplot (A) Doses 0.05:1 to 1:1 (B) Doses 5:1 to 20:1. (C) The percent change in cell count was calculated in each data set, using their respective controls.

Eosinophils Have a Broad Tumoricidal Effect

In order to investigate the breadth of eosinophil effect on cancer cells, subsequent experiments assessed the effect eosinophils had on three more cell lines, A549, RKO, and TF1 in addition to the original PC3 cell line. Eosinophils were incubated with tumor cells at various effector to target ratios for 4 hours before harvest. The eosinophil population was gated in order to exclude them from analysis, and cancer cell viability was determined by dual staining with Annexin V and PI.

Figure 3 shows the one A549 experiment that was conducted. This showed a drastic reduction of A549 viability with the lowest concentration, 5:1::eosinophils:A549, from the baseline 28% dead or dying cells to 80-90%.

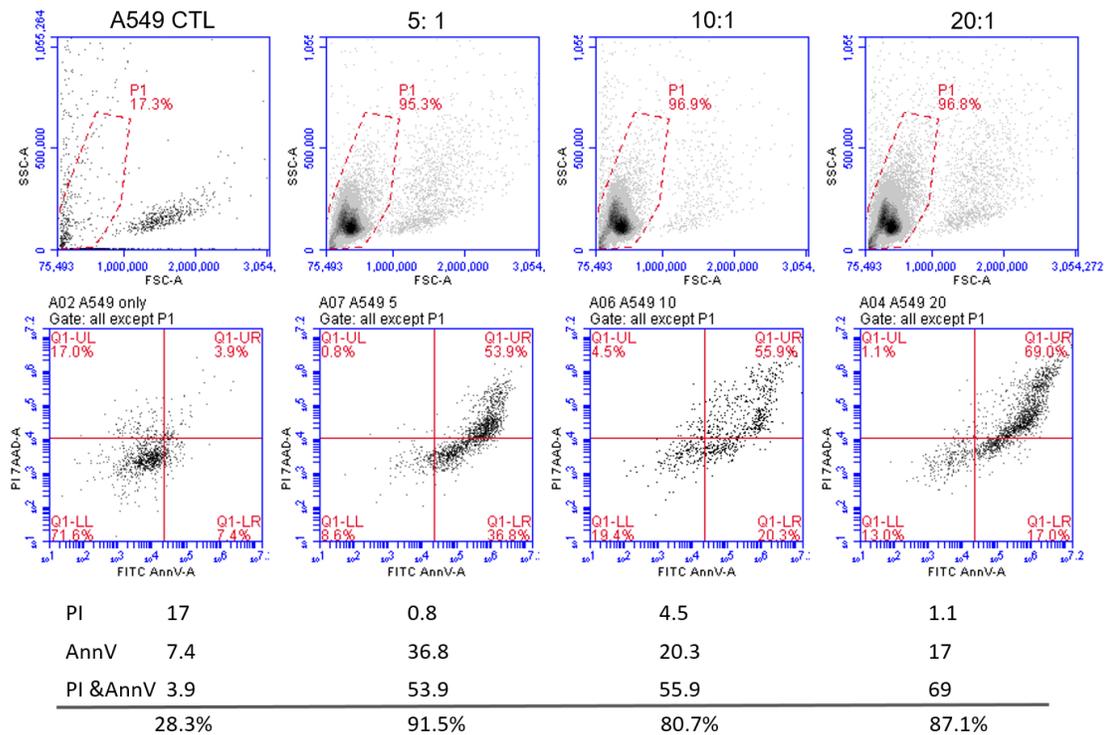


Figure 3. Flowcytometric Analysis of EOS:A549 Co-culture. A549 cancer cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs in the indicated ratios. The eosinophils were gated to exclude them from the analysis. Viability was measured as total positive for Annexin V or PI.

The RKO experiments initially showed cytotoxic effects of the eosinophil on RKO cells with an initial 11% dead or dying to 50-80% dead or dying (Figure 4). This was also seen when lower concentrations of eosinophils:RKO were added going from 19% dead or dying to 35% with the lowest concentration, 0.05:1, increasing up to 50% with the highest concentration, 20:1 (Figure 4). TF1s did respond to eosinophil treatment as well, but with less sensitivity to and in a less linear fashion. The initial experiment increased cytotoxicity from 33.7% to 40-50% (Figure 5), while the second experiment

showed no increase in cytotoxicity from the low concentration treatments. The higher doses did have a gradual concentration dependent increasing cytotoxicity from 18% to 44.7% (Figure 5).

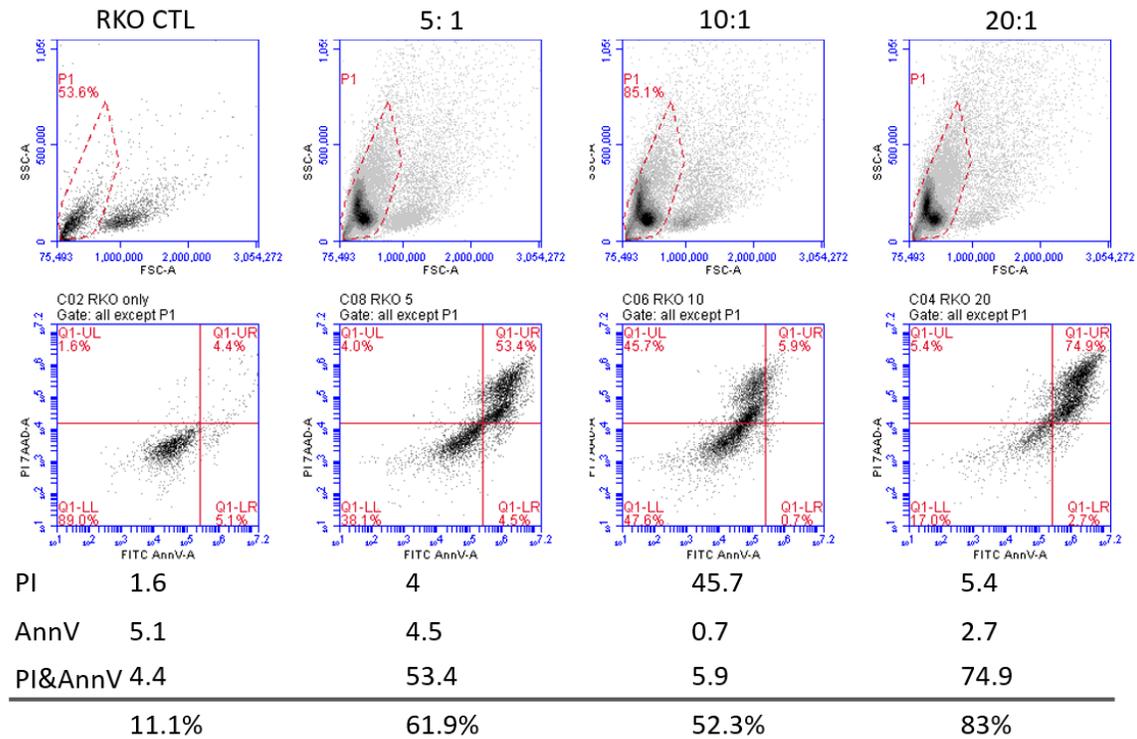


Figure 4. Flowcytometric Analysis of EOS:RKO Co-culture. RKO cancer cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs at ratios of 5:1, 10:1 and 20:1. eosinophils were gated to exclude them from the analysis. Viability was measured as total positive for Annexin V or PI.

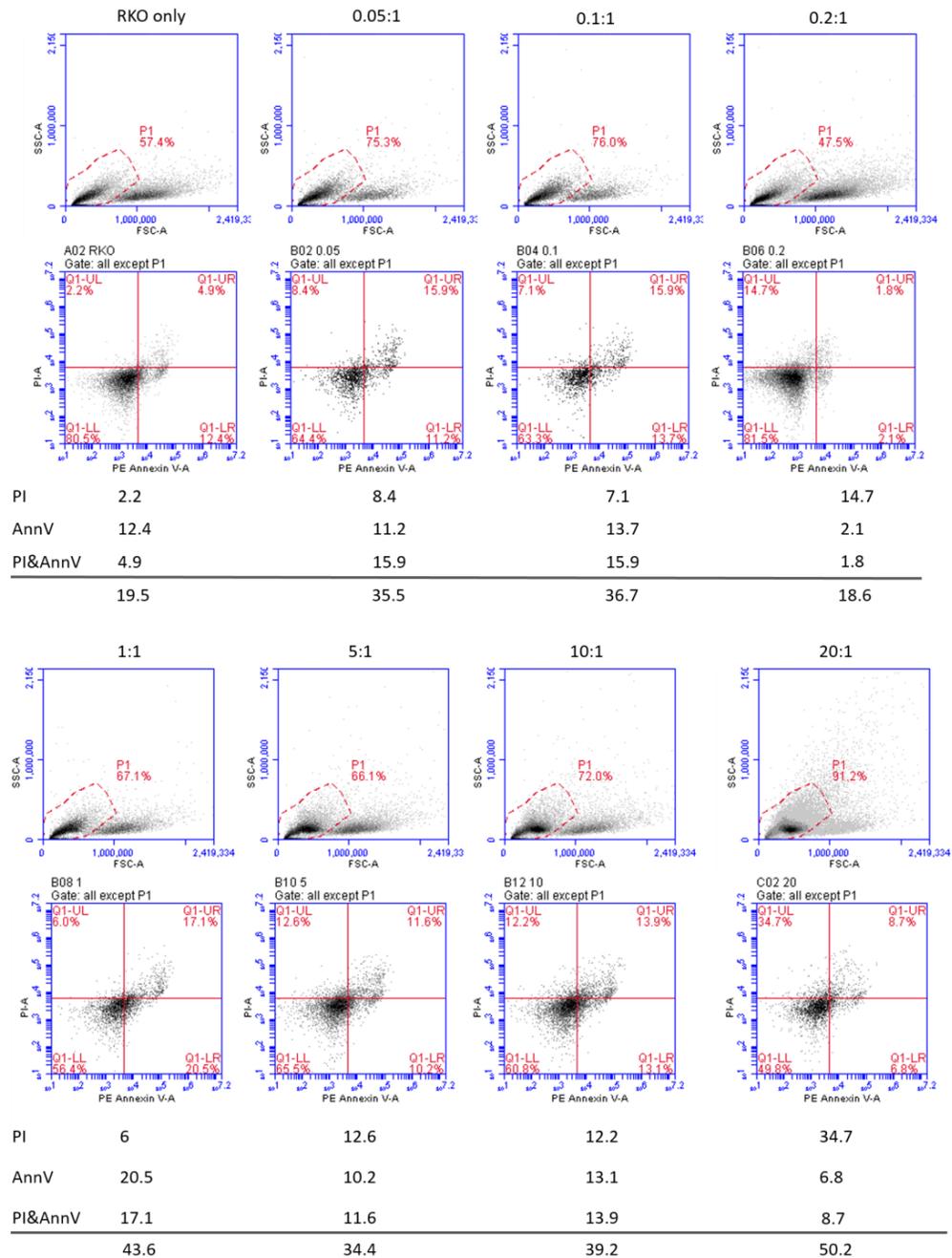


Figure 4 Continued. RKO cancer cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs concentrations of 0.05:1 - 20:1. The eosinophils were gated to exclude them from analysis. Viability was measured as total positive for Annexin V or PI.

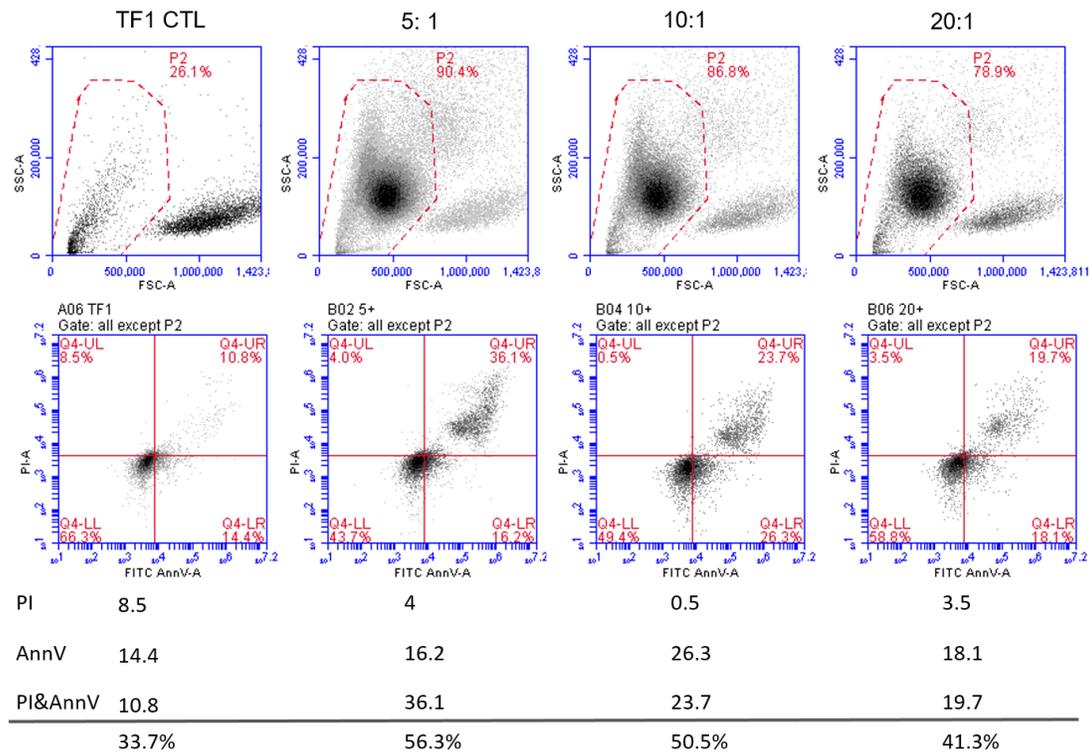


Figure 5. Flowcytometric Analysis of EOS:TF1 Co-culture. TF1 cancer cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured with TF1 cells for 4 hrs at ratios of 5:1, 10:1 and 20:1. The eosinophils were gated to exclude them from analysis. Viability was measured as total positive for Annexin V or PI.

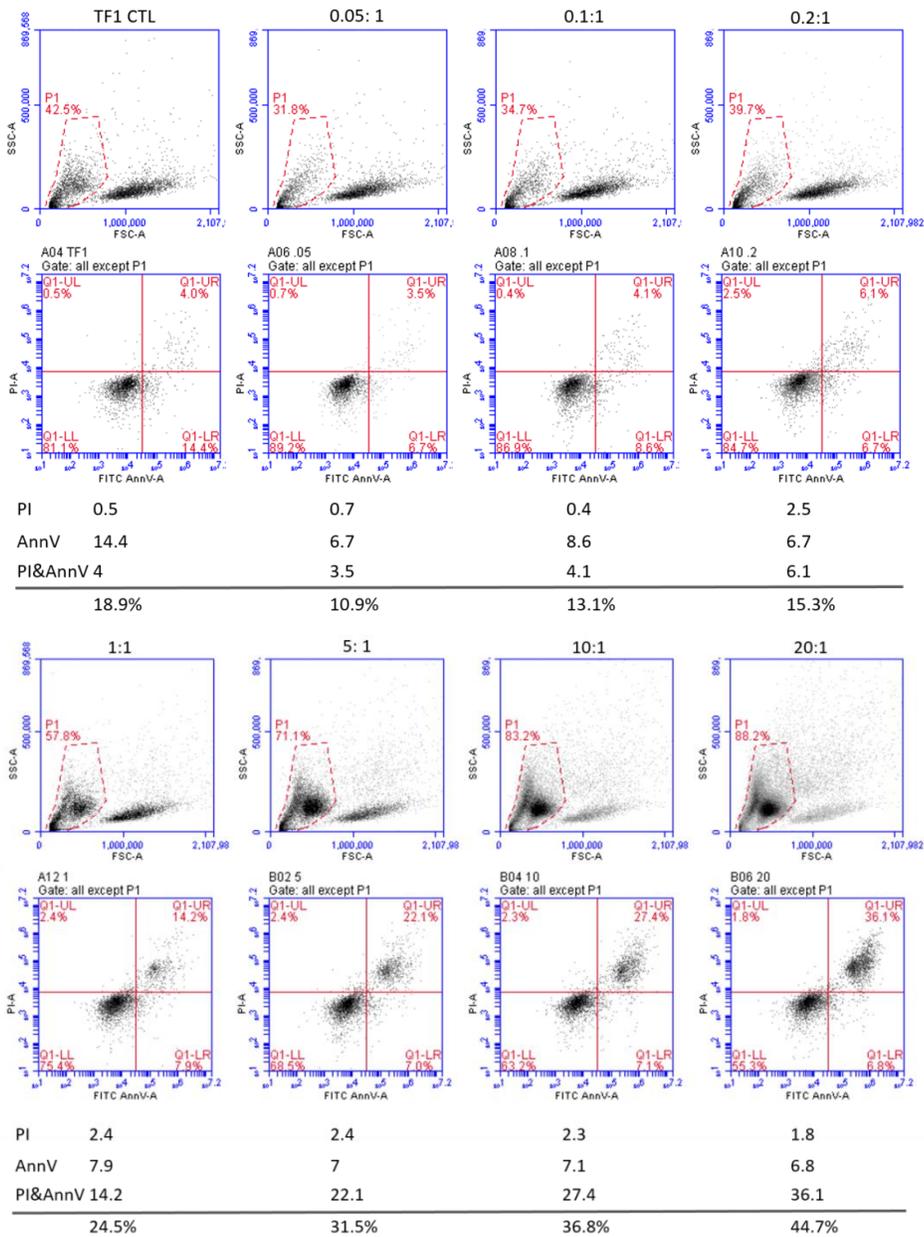


Figure 5 Continued. TF1 cancer cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured with TF1 cells for 4 hrs concentrations of 0.1:1-20:1 as indicated. Eosinophils were gated to exclude them from analysis. Viability was measured as total positive for Annexin V or PI.

The PC3 cell line was used in three experiments at varying eosinophil:PC3 concentrations with Figure 6B and 6C duplicates of the same experiment. PC3 cells seemed resistant to treatment with cytotoxic effects seen from 73% to 90% in the 5:1 and 10:1 concentrations, but unsustainable in the highest 20:1 concentration at 77% (Figure 6A). This trend was also seen in the latter experiment with increased cytotoxicity from 19% to 30-40% but decreased to 26-29% in the 20:1 concentration (Figure 6B and 6C).

From the aforementioned data, the percent of viable cells was extrapolated for comparison between cell lines (Figure 7). There was a cytotoxic effect on all cell lines with varying sensitivity with corresponding reduction in viability. A549 cells appeared to be the most sensitive followed by RKO, TF-1 and then PC3.

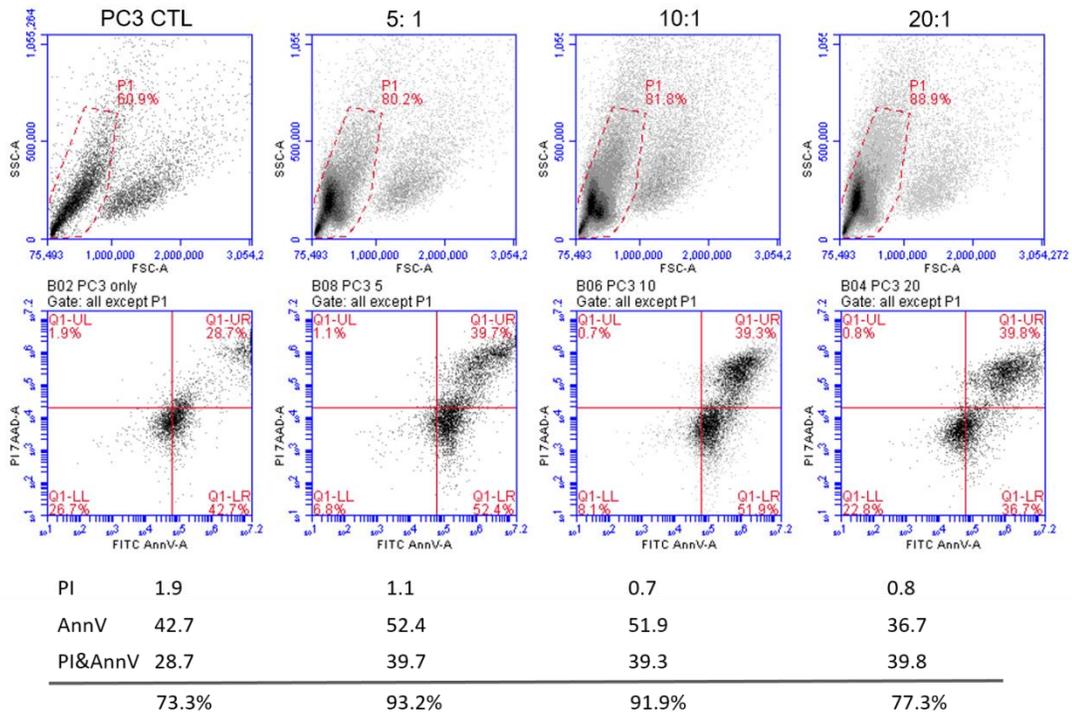


Figure 6. Flowcytometric Analysis of EOS:PC3 Co-culture. PC3 cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs concentrations of 5:1, 10:1 and 20:1. The cells were gated excluding the eosinophil population. Viability was measured as total positive for Annexin V or PI.

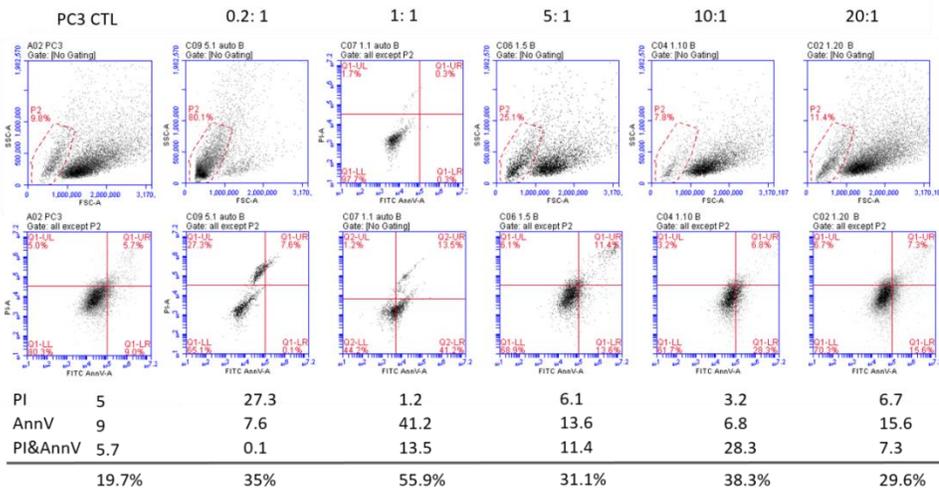
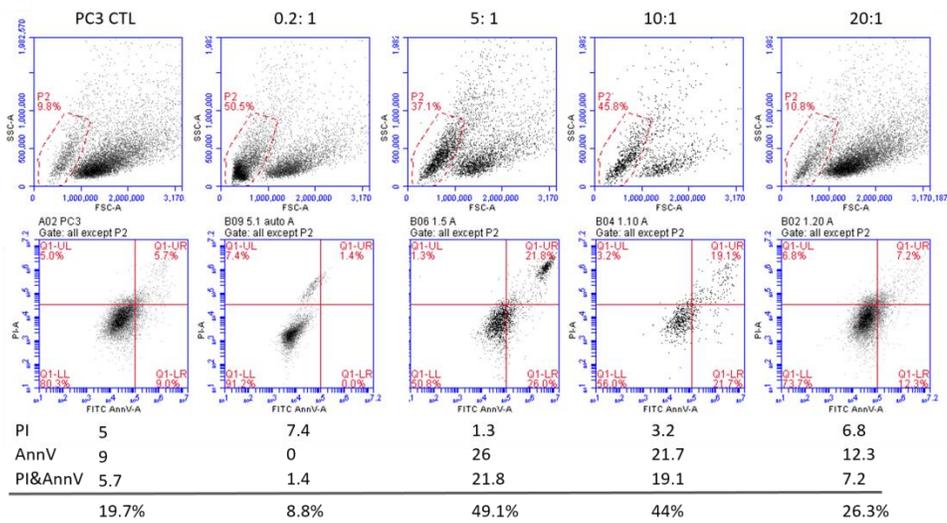


Figure 6 Continued. PC3 cells were plated on 96-well plates and incubated overnight. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF then co-cultured for 4 hrs concentrations 0.2:1- 20:1 as indicated. Eosinophils were gated to exclude them from analysis. Viability was measured as total positive for Annexin V or PI.

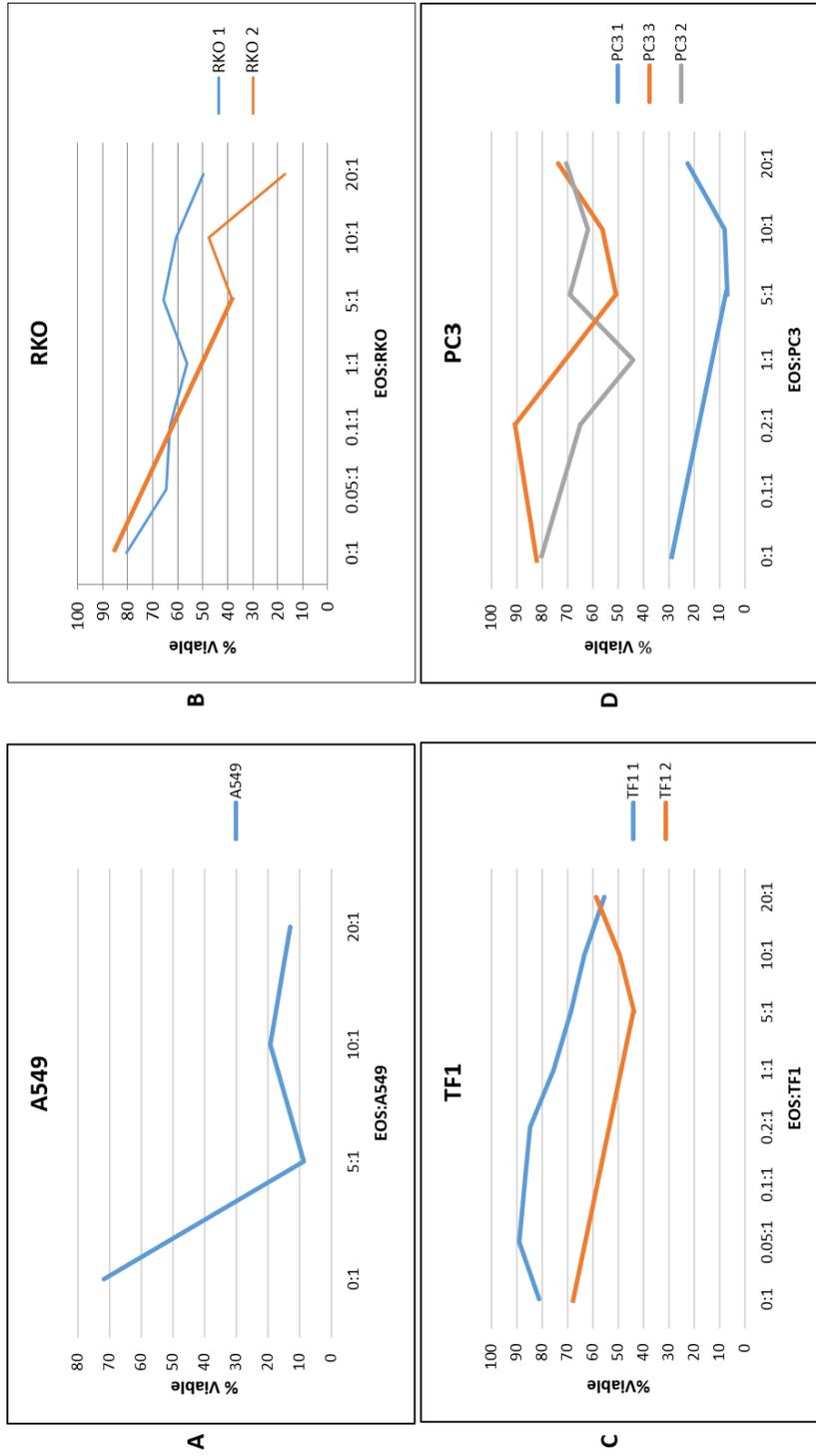


Figure 7. Eosinophils Have a Broad Tumoricidal Effect. Eosinophils were cultured overnight at a concentration of 1 million/mL in RPMI Media with 10% heat inactivated FBS and 10ng/mL of each IL-5 and GM-CSF. Cancer cells were plated in 96-well flat bottom plates overnight and eosinophils were added the next day at varying concentrations and co-incubated for 4 hours. Viability was measured with Annexin V and PI flow cytometry kit. (A) A549 NSLC cells (B) RKO colon cancer cells. Exp 1 and Exp 2 were two independent experiments performed with the indicated eosinophil doses. (C) TF1 erythroleukemic cells. Exp 1 and Exp 2 were two independent experiments performed with the indicated eosinophil doses. (D) PC3 prostate cancer cells. Exp 1-3 were independent experiments performed with the indicated eosinophil doses.

Discussion

Tumor associated eosinophilia is an established phenomenon with few studies illustrating the mechanism by which eosinophils and tumor cells interact. Our study demonstrated that eosinophils are tumoricidal when in co-culture with various human derived tumor cell lines. We have also demonstrated that different cancer cell types respond with different sensitivities to eosinophils in co-culture. In this paper we touched upon targets of cancer immunotherapy, which begs the question, what causes the eosinophil to be more effective against one cancer cell line than in another?

In our experiments, the A549 NSCLC cells were the most sensitive to treatment, followed by the other cancer lines, suggesting that eosinophils use pathways that better target NSCLC. While mutations in the epidermal growth factor receptor (EGFR) garnered much attention and are responsive to EGFR inhibitors like gefitinib and erlotinib, only 10% of lung cancer patients are EGFR+, limiting this treatment. Lung cancer is challenging to treat with traditional therapeutics due to their cancer stem cells (CSC) [111]. CSCs boast slow growth kinetics and resistance to ROS destruction due to high antioxidant levels. NSCLC becomes responsive therapy with CSC destruction. Studies have implicated *k-ras* and *βcatenin* as targets to help sensitize the cells to therapy with other regulators of stem cell expansion also having potential [112-116]. Future eosinophil studies concentrating on ROS targets and CSC may reveal the eosinophil's tumoricidal mechanism.

A problem we encountered was the inability to reliably separate the eosinophils and tumor cells. We initially thought that by gating the cancer cells and eosinophils that

there would be distinct populations to measure. However, we found that degranulating eosinophils and dying cancer cells could have overlap on the scatterplot. We attempted to treat this conservatively by gating and then excluding the eosinophil population, though this may have excluded some of our dead or dying cancer cells, hence underestimating the effect of the eosinophil treatment. Likewise, at high eosinophil:tumor cell concentrations, the degranulating eosinophils could have falsely elevated levels of Annexin V, overestimating the appearance of dead or dying tumor cells. Other modalities that could have been used include radiolabelling cancer cells and measuring the release of radioactive cell contents, excluding eosinophils by surface markers (CD16⁻CD66b⁺) and by downstream markers such as mitochondrial membrane potential, caspase production and proangiogenic factors.

CHAPTER III

EOSINOPHIL ACTIVATION

Given that TATE is well described in cancer patients, and can be associated with improved prognosis, the promotion of eosinophil effector function is a potentially viable strategy against tumors. To investigate the potential to enhance eosinophil tumoricidal activity, we compared tumoricidal activity of eosinophils cultured without or with known eosinophil agonists. Since the degranulation of eosinophils is key for eosinophil effector function, any strategy that would induce degranulation may be useful. The chemokine eotaxin can stimulate eosinophil degranulation, so eotaxin promotion might be beneficial. Indeed, an eosinophilotactic molecule was reportedly produced from a large-cell anaplastic carcinoma of the lung, so it may be possible to illicit a chemokine dependent response via modulation of the tumor [117]. Likewise, many cytokines are associated with eosinophil degranulation, including IL-5, IL-33, and GM-CSF [118, 119].

Measuring Eosinophil Activation

Eosinophil activation can be determined by measuring eosinophil degranulation reflected by protein expression in the plasma membrane and release of cytotoxic molecules. CD63, is a tetraspanin protein that is a component of the eosinophil crystalloid granule, has been shown to translocate to the plasma membrane due to eosinophil stimulation. Translocation has been induced by IL-5, GM-CSF, and IL-3 [120, 121].

CD69 is found in many hematopoietic cell lines and is upregulated by IL-3 in basophils [117, 122, 123]. It is a transmembrane protein with a C-lectin domain, which binds to extracellular matrix proteins to affect downstream signaling. CD69 is also expressed in eosinophils and has been used as an indicator of eosinophil activation. Downstream signaling is regulated by Jak3, STAT5, so STAT5pY could be used to measure eosinophil activation along with CD69 translocation to the plasma membrane.

Enhancing Eosinophil Activation

Studies have shown that IL-5 and GMCSF can induce eosinophil degranulation, so these maintenance cytokines may contribute to an eosinophil's cytotoxicity against tumor cells [125]. Both are necessary for hematopoietic stem cell differentiation into eosinophils. GMCSF is secreted by a number of different cell types including macrophages, mast cells, and endothelial cells, while also being necessary for directing neutrophil differentiation. IL-5 is secreted by Th2 and mast cells to stimulate eosinophil maturation and activation and is also secreted in an autocrine manner.

IL-33 has also been shown to mediate eosinophil adhesion and survival as well as degranulation [123,118]. It is expressed by epithelial cells, fibroblasts, and smooth muscle cells and mediates a Th2 response via the ST2 receptor.

Another strategy would be to cross link the surface receptors using an antibody specific to the receptor [126]. The eosinophil plays a role in antibody dependent immunology largely mediated through the immunoglobulin receptors Fc γ R, Fc α R, and Fc ϵ R, which are the IgG, IgA, and IgE Fc receptors respectively [127, 1268]. The use of protein A or G along with receptor specific antibodies could be used to crosslink

eosinophil cell surface receptors to initiate signaling cascades that promote degranulation. Interestingly, CD89, the Fc α R, must be bound by polymeric IgA in order to initiate the signaling cascade. When an IgA polymer binds, it leads to inhibition of the signaling cascade by partially phosphorylating the ITAMs and downstream molecules whereas full phosphorylation activates eosinophils (Figure 8). In order to activate Fc α R we formed an immune complex consisting of IgG anti-CD89 antibody and anti-IgG antibody.

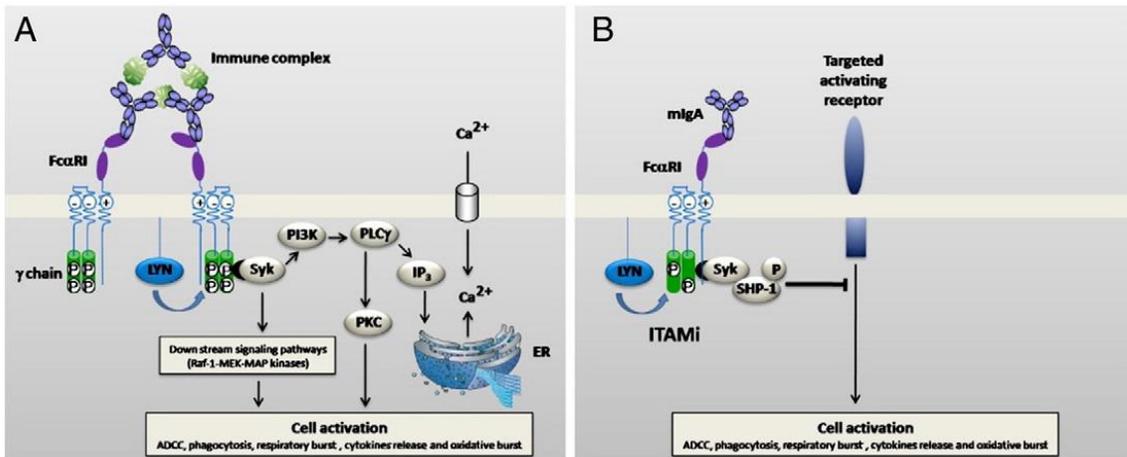


Figure 8. Regulation of Immune Response by Fc α R*. (A) ITAM-induced activation. Cross-linking of Fc α RI by immune complex results in full phosphorylation of ITAM tyrosines this leads to the phosphorylation of additional signaling adaptors which recruit several downstream molecules. Overall this induces immune cell activation with cell responses such as phagocytosis, cytokine synthesis, proliferation and degranulation. (B) ITAMi-homeostatic control. Monomeric IgA binding to Fc α RI results in partial phosphorylation of ITAM tyrosines resulting in SHP-1 recruitment. This would in time allow the long-lasting dephosphorylation of other signaling pathways.

*Reproduced from [110]

Another target is cell contact adhesion molecules that have been shown to play a role in eosinophil degranulation. As previously mentioned, CD11a and CD18 were

shown to be involved in eosinophil tumoricidal activity, as antibodies against these molecules attenuated the tumoricidal effect [10]. To investigate the role of cell contact in eosinophil activation, we followed Kita's protocol-coating culture plates with extracellular matrix proteins and blocking with human serum albumin before eosinophil incubation [129]. Besides those mentioned, other receptors targeted in prior studies include pattern recognition receptors, leukotriene receptors, and chemoattractant receptors [1, 18, 131].

Materials and Methods

Eosinophil Isolation and Culture

De-identified human blood Leukopak containing 50-70mL of blood were obtained from Gulf Coast Regional Blood Center and diluted 1:5 with HBSS, layered on Ficoll-Paque, and centrifuged 700 x g for 30 min at 18°C. The pellet was harvested and red blood cells were lysed and washed away and the remaining granulocytes were loaded into a Robosep with mAb negative selection for eosinophils, using an eosinophil purification mAb cocktail (StemCell Technologies).

Purity was measured by flow cytometer. Dual staining was performed with CD16 and CD66b. The eosinophil population is CD66b⁺ and CD16⁻, while a double positive population would be indicative of neutrophils. Purity was confirmed by light microscopy with a Wright-Giemsa stain. Cells of less than 90% eosinophil purity were not used for experimentation. Eosinophils were either used directly after isolation or incubated at 37°F overnight in RPMI media with 10% FBS at a concentration of 1 million cells /mL. Unless otherwise noted, cells incubated overnight were also treated with 10ng/mL GM-

CSF and IL-5, while some of the experiments also used other cytokines, discussed below.

Measuring Eosinophil Activation

We used FITC CD69 (BDPharmigen) and PE CD63 (Biolegend) as measures of eosinophil activation and degranulation. Isotype controls FITC mouse IgG1k and PE mouse IgG1k respectively.

Results

Effect of Cytokines on Eosinophil Activation and Receptor Expression

Baseline expression of IL-3R α , IL-5R α , GM-CSFR α and β C was measured to be able to compare in later experiments should our treatments affect receptor expression. Eosinophils were isolated and flow cytometry was used to measure presence of the receptors. There was no baseline expression of IL-3R α while there was a basal expression of IL-5R α , the β C chain, CD89-the IgA receptor, and ST2-the IL-33 receptor (Figure 9). Cells were then incubated overnight with media or one of the following cytokines: IL-3, IL-5, IL-33, or GM-CSF (Figure 10). IL-5R α and the common beta C chains were increased with all treatments while IL-3R α , and GM-CSFR α had no increased expression following any of the cytokine experiments.

This experiment also measured the effect of cytokine incubation and eosinophil activation (Figure 11). We saw that CD69 expression was not increased with overnight incubation while CD63 expression was increased with treatment of IL-3 and GM-CSF with a smaller activating effect from IL-5 and IL-33.

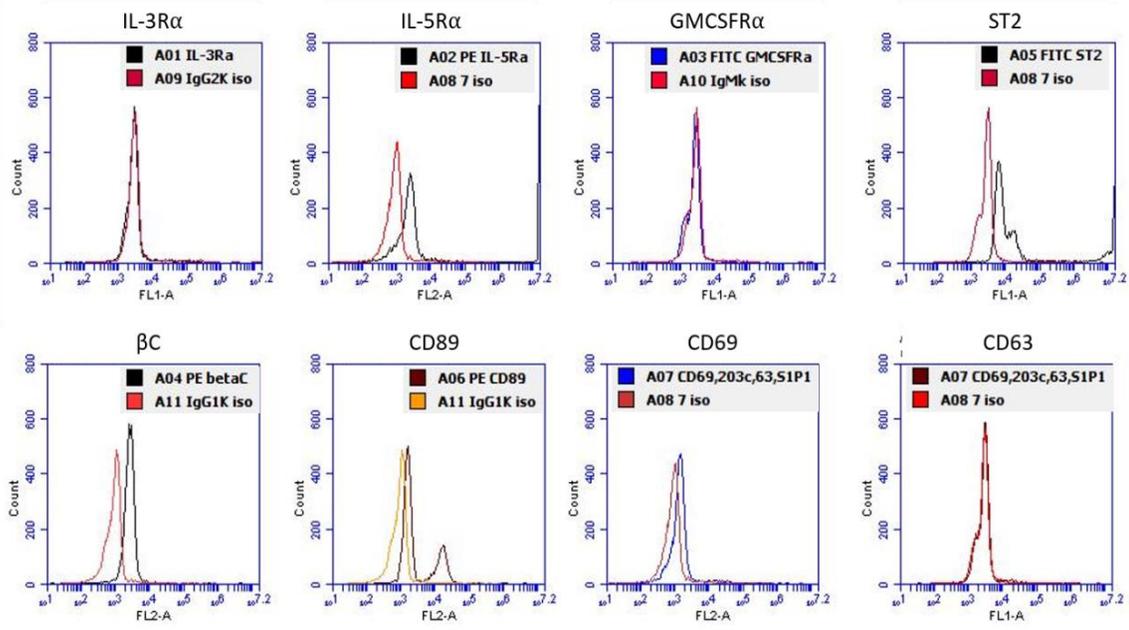


Figure 9. Baseline Receptor Expression. Freshly isolated eosinophils were incubated with antibodies to the indicated receptors. Receptor expression was measured via flow cytometry.

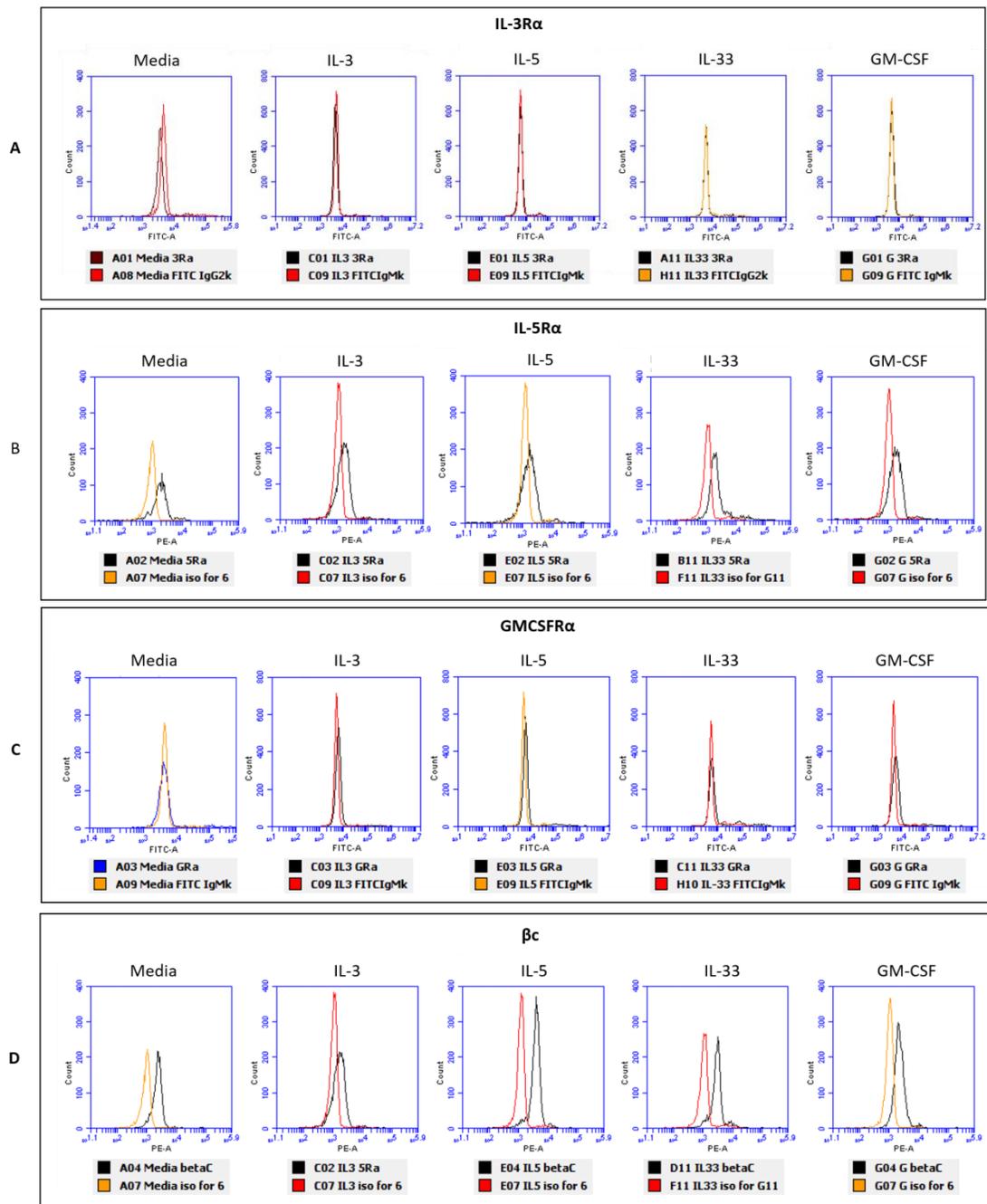


Figure 10. Cytokine Treatment and Eosinophil Receptors. Eosinophils were plated in 24 well plates and incubated overnight with 10ng/mL IL-5, IL-3, IL-33 or GM-CSF. Expression of (A) IL-3R α , (B) IL-5R α , (C) GM-CSFR α and (D) β c were measured via flow cytometry.

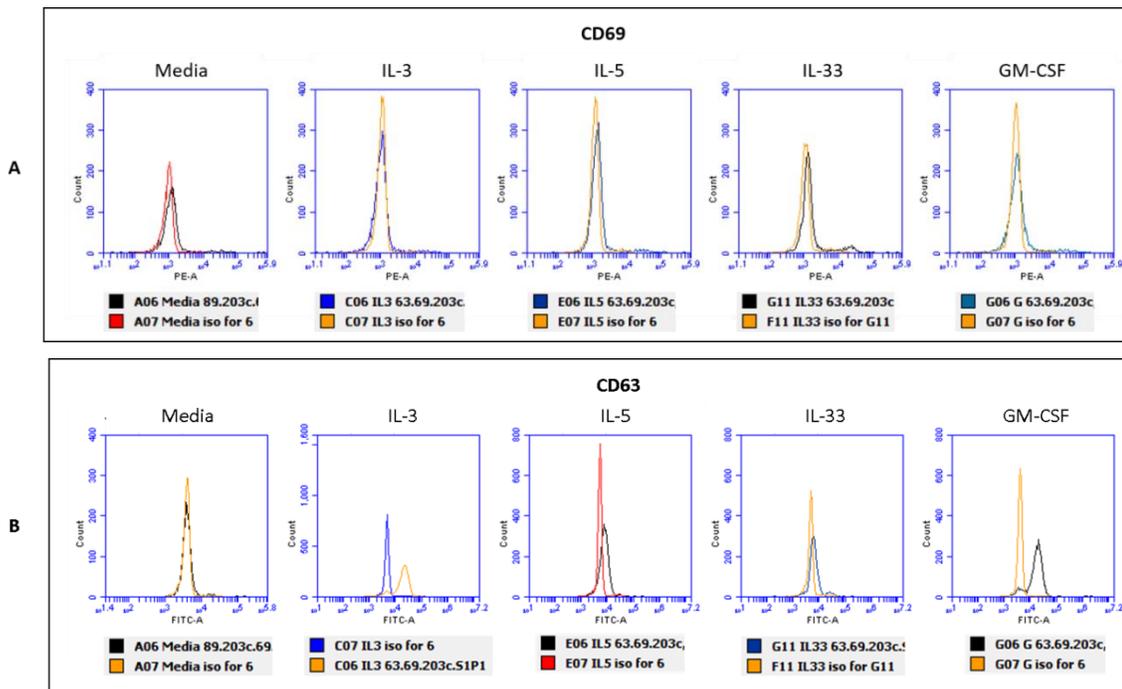


Figure 11. Cytokine Treatment and Eosinophil Activation. Eosinophils were plated in 24 well plates and incubated overnight with 10ng/mL IL-5, IL-3, IL-33 or GM-CSF. CD63 (A) and CD69 (B) were measured by flow cytometry.

IL-33 Increases CD63 Expression

Similarly, acute incubation of eosinophils with IL-33 induces CD63 expression. Eosinophils were freshly isolated as previously described and treated with 5ng/mL IL-5 and GM-CSF overnight. The eosinophils were treated with IL-33 for 3.5 hours before harvest and eosinophil degranulation was measured by CD63 expression. Eosinophils had 10% more CD63 positivity when treated with IL-33 compared to control (Figure 12).

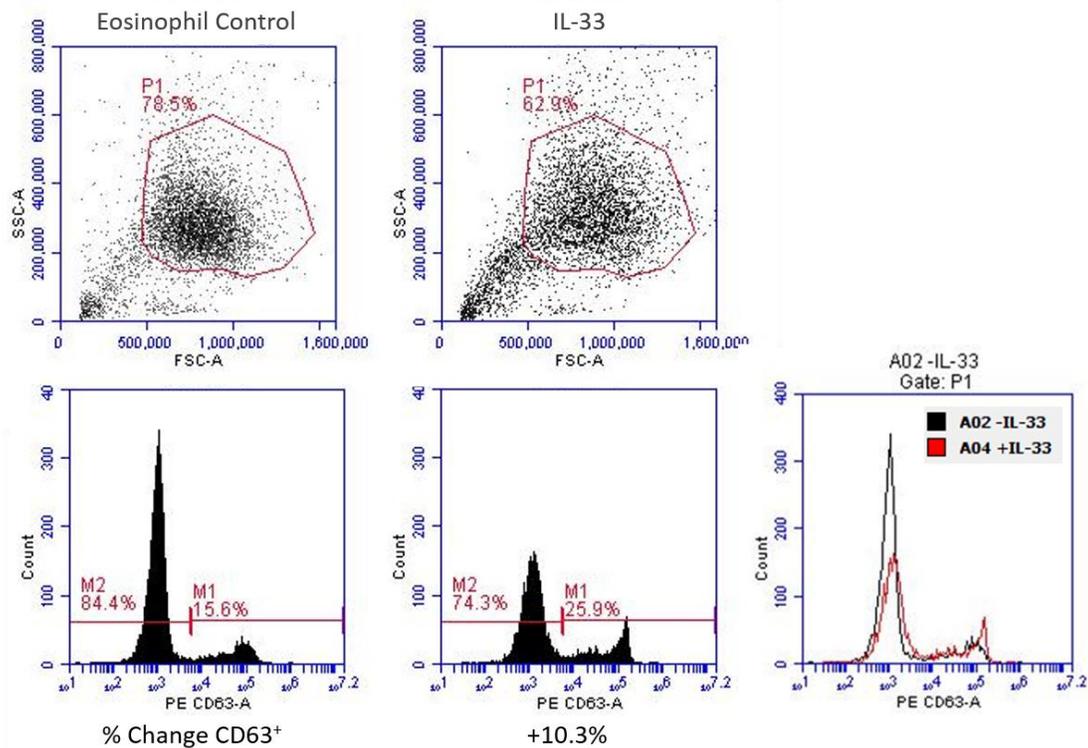


Figure 12. IL-33 Increases CD63 Expression. Eosinophils were freshly isolated and plated in 96-well plates and then treated with IL-5 and GM-CSF overnight and then with media (CTL) or IL-33 for 3.5hrs. CD63 expression was analyzed via flow cytometry.

IL-5 and GM-CSF Increase CD63 Expression

We also measured cytokine eosinophil activation in the presence of anti-CD89mAb to see if that would enhance the effect. Eosinophils were freshly isolated as previously described and treated with media, IL-5 or GM-CSF overnight. The eosinophils were treated with anti-CD89mAb 1 hour before harvest and eosinophil degranulation was measured by CD63 expression. Eosinophils had greater activation in response to GM-CSF than when treated with IL-5. When treated with both IL-5 and GM-CSF there was activation greater than IL-5 alone, but less than GM-CSF alone

(Figure 13), indicating that IL-5 prevents degranulation of eosinophils attenuating the effect of GM-CSF.

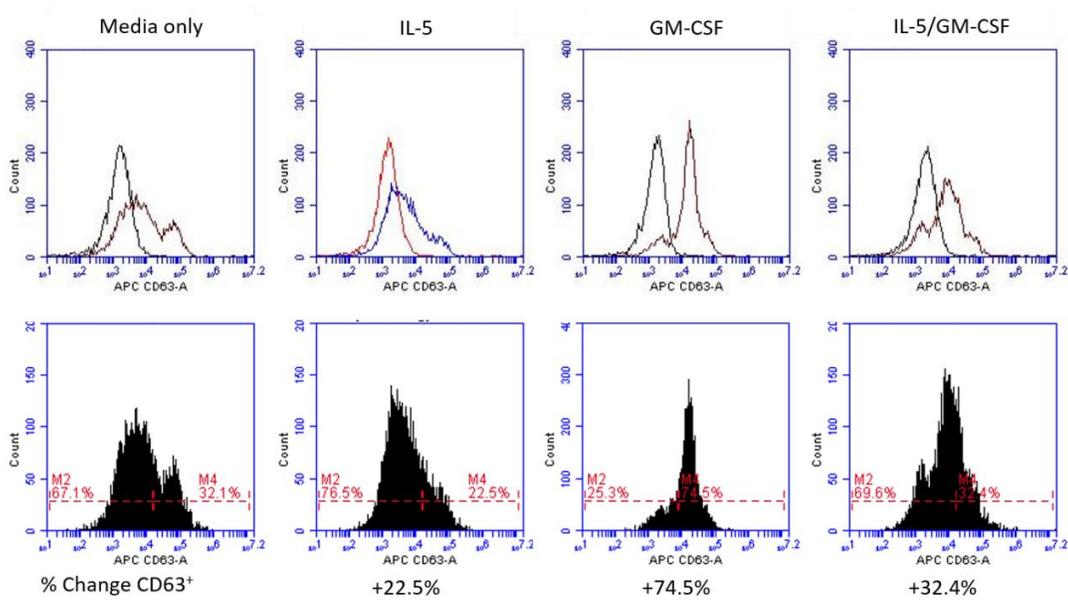


Figure 13. IL-5 and GM-CSF Increase CD63 Expression. Eosinophils were freshly isolated and plated in 24-well plates and then treated with media control or respective cytokines. The next morning they were treated with 100ng/mL anti-CD89 mAb for 1 hour and CD63 expression was analyzed via flow cytometry.

Effect of Cytokines and anti-CD89mAb on Eosinophil Receptors

Eosinophils were incubated overnight with 10ng/mL IL-5, IL-3, IL-33 or GM-CSF, and then incubated for 1 hour with 100ng/mL anti-CD89mAb. Flow cytometry was used to measure expression of IL-3R α , IL-5R α , GM-CSFR α and β C. IL-3R α and GMCSFR α did not have increased expression on any of the treatment groups, while IL-5R α , β c-R and were expressed equally on all treatment groups (Figure 14). Eosinophil activation was measured using CD69 and CD63 via flow cytometry (Figure 15). CD69 expression was unchanged, while CD63 expression was increased in all cytokine and cross-linked cells, with the highest expression in those treated with IL-3, GM-CSF, and IL-5 respectively.

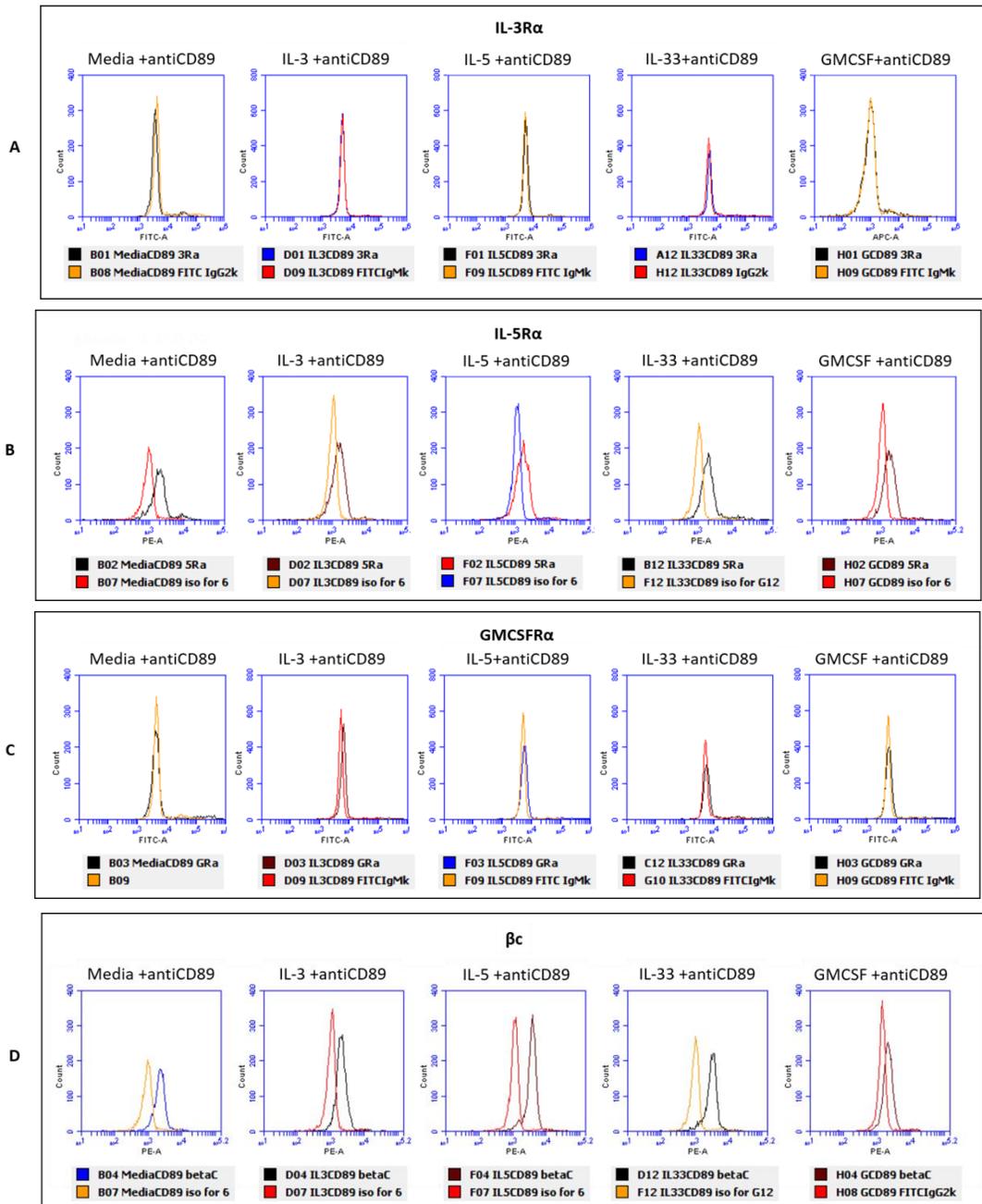


Figure 14. Cytokine Treatment, CD89 and Eosinophil Receptors. Eosinophils were plated in 24 well plates and incubated overnight with 10ng/mL IL-5, IL-3, IL-33 or GM-CSF. And then treated with anti-CD89mAb. Expression of (A) IL-3 α , (B) IL-5R α , (C) GM-CSFR α and (D) β c were measured via flow cytometry.

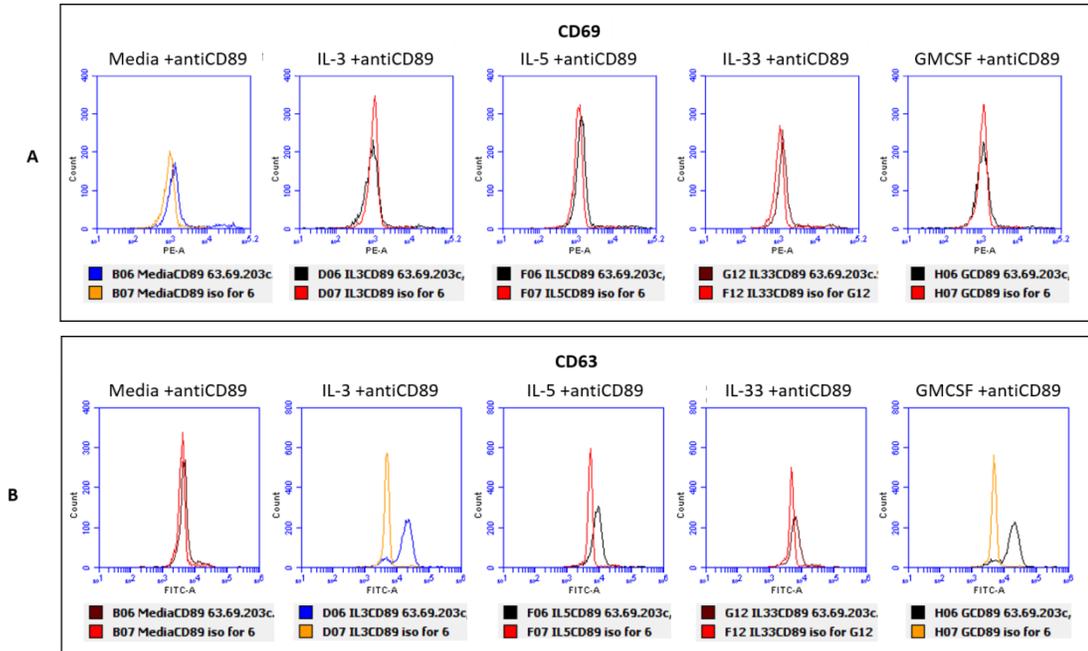


Figure 15. Cytokine Treatment, CD89 and Eosinophil Activation. Eosinophils were plated in 24 well plates and incubated overnight with 10ng/mL IL-5, IL-3, IL-33 or GM-CSF, then incubated for 1hr with anti-CD89mAb. CD63 (A) and CD69 (B) were measured by flow cytometry.

CD63 Expression Is Increased with anti-CD89mAb

Fresh eosinophils were isolated and incubated with 10ng/mL anti-CD89mAb for 30 min or incubated overnight with 5ng/mL IL-5 and GM-CSF and the next morning treated with anti-CD89mAb for 30 minutes (Figure 16A). Activation was measured via CD63 expression on flow cytometry. CD63 expression increased when freshly isolated eosinophils were treated with anti-CD89mAb. Eosinophils treated overnight had no increase in activation as measured by CD63 expression (Figure 16B).

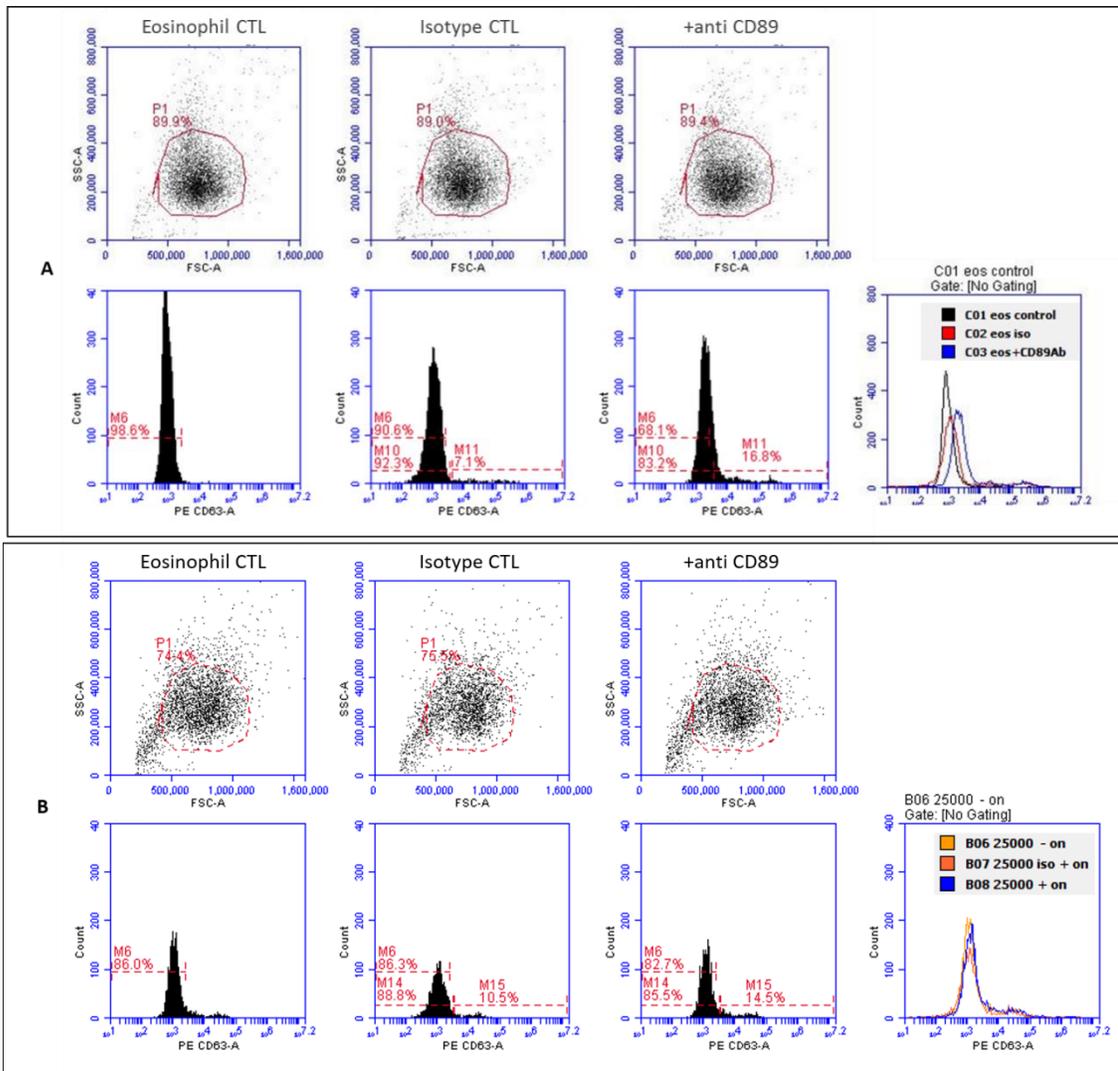


Figure 16. CD63 Expression Is Increased with anti-CD89mAb. Eosinophils were isolated and incubated with 10ng/mL anti-CD89mAb then stained for CD63 on flow cytometry. (A) Fresh eosinophils were treated for 30 minutes. (B) Eosinophils were incubated overnight with 5ng/mL IL-5 and GM-CSF and treated with anti-CD89mAb 30 minutes before harvest.

Anti-IgGmAb Increased CD63 Expression

Eosinophils were isolated and incubated overnight before treatment with IL-33, then with anti-CD89mAb, followed by incubation with anti-IgGmAb. In contrast to previous experiments, IL-33 treatment alone has a small effect on eosinophil degranulation, while anti-CD89mAb has no effect on CD63 expression. Together, those eosinophils treated with IL-33, anti-CD89mAb and anti-IgG1mAb did increase CD63 expression (Figure 17).

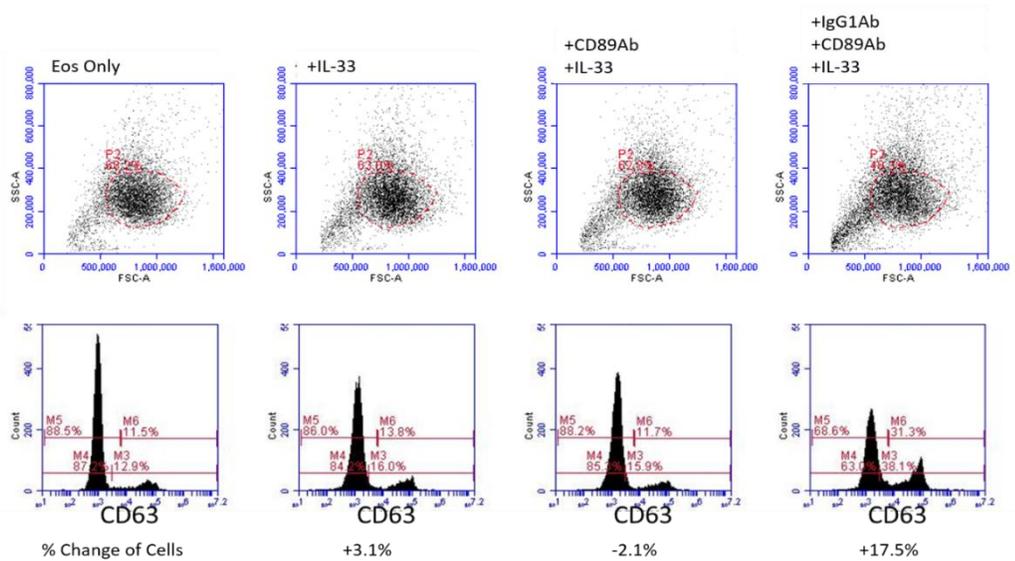


Figure 17. Anti-IgGmAb Increases CD63 Expression. Eosinophils were incubated overnight with 10ng/mL IL-5 and GM-CSF. Then 1 hour treatment of IL-33, 1 hours treatment anti-CD89mAb, and 1 hour treatment anti-IgGmAb. CD63 expression was measured via flow cytometry. Isotype controls were used and found to be comparable to the eosinophil control.

Discussion

The main goal of this set of experiments was to use known activators of eosinophil degranulation to enhance degranulation and to target those that could affect tumor survival. Our experiments concentrated on reproducing these experiments to harness them in co-culture with tumor cell lines. We demonstrated that overnight treatments of IL-5, GM-CSF, IL-33. IL-33 activated eosinophils and this was confirmed with an acute incubation. Likewise, we showed that IL-5 and GM-CSF, in the presence of anti-CD89mAb, induced CD63 expression. Of note, there was a greater expression of CD63 in the GMCSF population with a 74% positivity in contrast to the 22% positivity. We also saw that there was not a synergistic effect of the two cytokines together with 33% CD63 positivity when treated with both IL-5 and GM-CSF. This indicates that IL-5 dampens degranulation. It is likely that IL-5 acted to stabilize the eosinophil against degranulation, acting as a pro-survival cytokine in this instance.

We were able to utilize principles of previous studies to show that eosinophils could be activated by crosslinking the CD89 receptor with anti-CD89mAb as well as with anti-IgGmAb. Unexpectedly, incubating the two together did not amplify CD63 expression, and when we treated with IL-33 we did not see the shift in CD63 that we had previously seen.

As we used donor human eosinophils, the environments they were previously in could affect their molecular milieu giving a heterogeneous eosinophil population. It is difficult to interpret the data accurately, so repeat studies would best measure the internal validity of these experiments.

Alternative ways to measure eosinophil activation include downstream indicators of activation such as phosphorylated signaling molecules, RNA transcription and protein production. Granule contents like EDN, ECP, ROS and MBP could be measured by ELISA.

Not shown here are two experiments we were unable to analyze. The first was to create an IgA immune complex consisting of anti-CD89 and Protein G. We did not account for the size of Protein G, and our flow cytometer could not process the results. In another experiment we used collagen to reproduce a cell-contact mediated eosinophil activation. Our control sample did not process appropriately, so we were unable to analyze our treatment groups.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The potential to harness eosinophils against cancers is an exciting frontier that could potentially lead to technological breakthroughs in clinical medicine. Our studies showed that when co-cultured, human eosinophils are tumoricidal to human-derived cancer cell lines. One oversight was that we did not provide a negative control demonstrating the specificity of eosinophil cytotoxicity to tumor cells. We will repeat our experiments use a non-transformable, terminally differentiated cell line to address this lapse. Once we show eosinophils' cytotoxicity to tumor cells we will conduct more studies to fully explore the modalities to harness eosinophil cytotoxicity in order to optimize effector to target cell ratios. We plan to continue our co-culture experiments and remove factors to hone in on the mechanism involved. For cell contact, we propose using plates with basket inserts to see if tumor death occurs in the absence of cell contact. To look at the various cytokines we could treat the eosinophils with various combinations of cytokines and neutralizing antibodies before co-culture to see how the cancer cells are affected along with better looking at eosinophil activation and culture with and without cytokines. We could also neutralize eosinophil granule contents. ROS are an attractive target as they have known implications for tumor survival, so we plan to use them as a measure of cytotoxicity along with Annexin V and PI to measure viability. We also plan to inhibit ROS production via glutathione to strengthen the link. The role of eosinophilia in tumor progression is still unknown, but future studies may provide us the insight to harness eosinophils as a cancer immunotherapy.

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