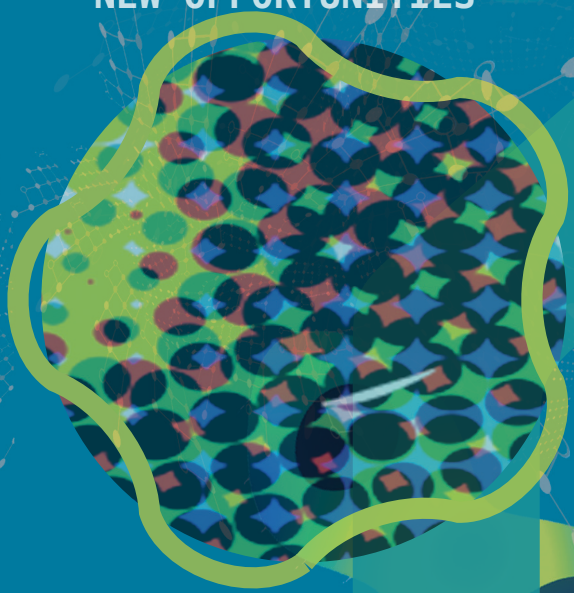
Breaking barriers: unraveling response mechanisms to immunotherapy in breast cancer

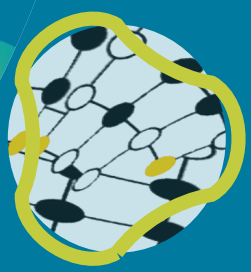
Blomberg, O.S.

RESPONSE

RESISTANCE

NEW OPPORTUNITIES





Discussion

Cancer is one of the leading causes of death worldwide. Despite major advances in the treatment of breast cancer, patients with advanced metastatic disease are, with rare exception, incurable by current treatment options. Throughout the process of cancer initiation, tumor progression and metastatic spread, cancer cells interact with the immune system. While some immune cells, when properly primed and activated, inhibit or kill cancer cells, others are hijacked by the cancer to facilitate immune evasion, tumor progression and metastasis[1]. The tumor's fate ultimately depends on the balance between anti-tumor immunity and tumor-promoting inflammation.

The goal of immunotherapy is to tip this balance in favor of anti-tumor immunity. Immune checkpoint blockade (ICB), such as the inhibitors of PD-1 and CTLA-4 used predominantly in this thesis, aims to improve the priming, expansion and effector functions of tumor specific $CD8^+$ T cells[2]. While $CD8^+$ T cells are important final effector cells in anti-tumor immunity, they do not act alone. They rely on other immune cells for proper activation and recruitment to the tumor. Conversely, they are also hampered via a plethora of immunosuppressive mechanisms employed by the tumor itself as well as by a range of other immune cells that are present in the tumor microenvironment as well as the systemic immune milieu. In this thesis, I aimed to unravel the immune complexity behind immunotherapy response in primary and metastatic breast cancer. The main questions I addressed are:

1) What are the cellular determinants for response and resistance to immunotherapy in breast cancer?
2) How can we rationally exploit these mechanisms to improve immunotherapy response in breast cancer?

In **chapter 2** we describe the complex multistep process of metastasis formation and examine the dual role of the immune system herein. We focus particularly on the interactions between cancer cells and immune cells and the reciprocal crosstalk among different immune cell populations. Through mechanistic insights in immune regulation of metastasis, we shed light on how these interactions may be therapeutically exploited to combat metastasis. In **chapter 3** we set out to study the mechanisms of immune checkpoint blockade response in primary and metastatic breast cancer by combining immunophenotyping in breast cancer patients with mechanistic studies in breast cancer mouse models. We discovered an unexpected player in anti-tumor immunity: the eosinophil. We propose that therapeutic engagement of eosinophils may be a way to improve immunotherapy responses in the future. In **chapters 4** and **5** we examined the negative regulators of anti-tumor immunity and immunotherapy response. Specifically, we investigated the role of immunosuppressive regulatory T cells (T_{regs}) (**chapter 4**) and neutrophils (**chapter 5**). We demonstrate that interference with these tumor-induced immunosuppressive players can enhance immunotherapy efficacy in breast cancer. In **chapter 6** we investigated whether PD1-IL2v, a new immunomodulatory agent that shows promise in pancreatic cancer models[3-5], may be suitable for use in breast cancer by putting it to the test in our lowly immunogenic KEP mammary tumor model. We discovered that combination treatment with cisplatin is a powerful approach to induce a broad activation of systemic and intratumoral adaptive and innate immunity, resulting in effective immunotherapy responses.

In this final **chapter**, I discuss the key mechanisms of the response and

resistance to immunotherapy in breast cancer we identified in our research emphasizing the importance of innate - adaptive immune cell crosstalk in the tumor micro- and macro-environment that underlie these processes. I put our findings in the context of existing literature and discuss future research directions and potential new therapeutic strategies.

Eosinophils; a new ally in anti-tumor immunity

Through unbiased profiling of the systemic immune landscape upon ICB in patients with metastatic triple-negative breast cancer (TNBC) and mechanistic studies in clinically relevant mouse models of primary and metastatic breast cancer, we identified eosinophils as unexpected players in immunotherapy response (**chapter 3)**. Eosinophils are bone marrow derived granulocytes that have been extensively studied for their role in tissue homeostasis and repair, parasite clearance and the pathophysiology of various diseases, including allergic asthma and autoimmunity[6]. However, in the context of cancer, they had been largely overlooked. With the publication of some intriguing reports over the last few years, eosinophils are now gaining increasing attention[7]. Eosinophils have been shown to infiltrate many tumor types, albeit to varying degrees[8], and are now recognized as integral parts of the tumor microenvironment (TME). Nevertheless, their role in cancer remains controversial, with opposing functions being reported depending on cancer type and disease stage[9-18]. The first indications that eosinophils may have a role in immunotherapy response came from correlative studies demonstrating that increased eosinophil counts during ICB treatment is associated with response to PD-1, PD-L1 or CTLA-4 targeting antibodies in patients with metastatic melanoma[19-21], non-small cell lung cancer (NSCLC)[22,23] and renal cell carcinoma (RCC)[24]. However, the question remained whether eosinophils merely serve as a biomarker for or were causally involved in ICB response. Our data in **chapter 3** add to this existing body of literature demonstrating that a systemic eosinophil increase is also associated with ICB response in patients with metastatic TNBC. Moreover, we uncovered that eosinophils play a critical role in immunotherapy response by promoting $CD8^+$ T cell activation in the tumor. Our mechanistic studies elucidated the mechanism by which ICB therapy induces systemic eosinophil expansion and tumor infiltration, a process which is initiated by IL-5-producing $CD4^+$ T cells upon ICB-induced activation and further driven by IL-33 expression upon combined cisplatin and ICB treatment. Our data highlight the importance of the reciprocal interactions between eosinophils and different T cell populations that are required for each other's recruitment and activation, emphasizing the interconnectivities between innate and adaptive immune cells during effective immunotherapy response. In addition, we were able to validate the different elements of the mechanism identified in our preclinical models in breast cancer patient samples, underscoring the power of combining translational research in cancer patients with mechanistic studies in clinically relevant mouse models to uncover novel mechanisms of immunotherapy response.

The mechanisms we identified draw many parallels with eosinophil biology in inflammatory diseases such as asthma[25]. $CD4^+$ T cells are key mediators of eosinophil recruitment to the asthmatic lung via secretion of IL-5[26,27]. In turn, eosinophils are not only effector cells of innate immunity, but they also have versatile

immunoregulatory functions controlling adaptive immune responses via antigen-presentation[28] and the regulation of T cell recruitment and Th1 and Th2 T cell polarization[29-31]. Through our work and that of others, we now know that eosinophils contribute to anti-tumor immunity through a similar variety of mechanisms, exerting direct tumoricidal effects[14] or enhancing anti-tumor immunity via changing the vasculature or re-shaping the tumor immune landscape, affecting both $CD8^+$ T cell recruitment and activation[11-13,32]. Our study in *Keratin14-Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (KEP) mammary tumors demonstrated that eosinophils predominantly promote $CD8^+$ T cell activation, rather than recruitment. However, we did not identify the precise mechanism through which eosinophils enhance $CD8^+$ T cell activation. Our more recent experiments provide some tantalizing clues. Assessment of the expression of T cell recruiting chemokines in the TME of KEP mice revealed that CIS + ICB therapy increased CXCL9 levels in the tumor compared to control-treated mice, which was lost upon eosinophil depletion using anti-SiglecF (**Figure 1A**), suggesting that eosinophils enhance CXCL9 production in KEP tumors. Blockade of CXCL9 prevented CIS + ICB induced $CD8^+$ T cell activation (**Figure 1B**), without affecting $CD8^+$ T cell infiltration (data not shown), suggesting that CXCL9 is involved in promoting $CD8^+$ T cell activation upon CIS + ICB therapy. Whether eosinophils produce CXCL9 directly or indirectly by stimulating the production of CXCL9 in other tumor-infiltrating immune cells, remains a topic of future research (**Figure 1C**).

CXCL9 expression in tumors was identified as the most conserved feature of response to checkpoint inhibition across cancer types, in addition to tumor mutational burden[33]. In patients with ER-negative breast cancer, expression of CXCL9 correlates to increased lymphocyte infiltration and improved overall survival[34]. Although traditionally linked to $CD8^+$ T cell recruitment, recent studies point out a role for CXCL9 in T cell activation as well[35]. Eosinophils have been shown to produce, amongst others, CXCL9 upon *in vitro* stimulation with IFNγ and TNFα and eosinophil expression of these chemokines was linked to T cell recruitment to B16 melanoma tumors[12]. However, eosinophils may also indirectly stimulate the production of CXCL9 in the TME through some unknown intermediaries. Both $CD103^+$ dendritic cells (cDC1s) and tumor-associated macrophages (TAMs) have been described to be critical producers of CXCL9 in the TME, required for T-cell infiltration and tumor control upon adoptive T cell transfer and immune checkpoint blockade, respectively[36,37]. Although adipose-tissue eosinophils are important for maintenance of alternatively activated (M2-like) macrophages involved in glucose metabolism[38], in the context of cancer, eosinophils may also affect macrophage reprogramming towards anti-tumorigenic phenotype[12]. In allergic airway inflammation, eosinophils play a role in the activation of dendritic cells, which, in turn, promote T cell activation[29-31]. Assessment of CXCL9 protein levels in sorted tumor-infiltrating eosinophils and other myeloid cell populations upon CIS + ICB therapy in KEP mice, is required to provide a definitive answer.

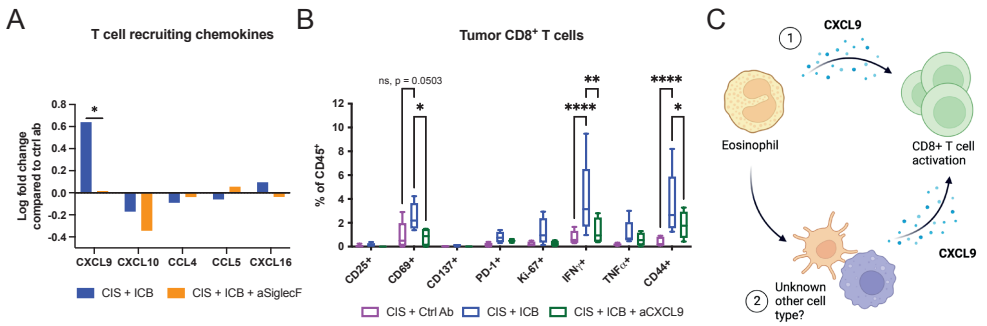


Figure 1. Eosinophils mediate T cell activation upon CIS + ICB in a CXCL9-dependent manner
(A) Assessment of T cell recruiting chemokines in tumor lysates of KEP tumors measured by LegendPlex at day 21 after start of indicated treatment, relative to control treated. Unpaired t-test. (B) Frequency of indicated activation markers expressed on intratumoral CD8⁺ T cells upon different treatments, determined by flow cytometry (n=4-5). Boxes represent median and interquartile range, whiskers full range. Two-way ANOVA followed by Tukey's multiple comparison test. (C) Schematic representation of the potential ways that eosinophils mediate CD8⁺ T cells activation in the tumor in an CXCL9-dependent manner. Eosinophils may (1) directly produce CXCL9 or (2) indirectly stimulate the production of CXCL9 in other tumor-infiltrating immune cells such as in TAMs or DCs. ns, not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Phenotypical and functional diversity of eosinophils

Eosinophils were traditionally considered terminally differentiated cells once they leave the bone marrow, but this notion is shifting in recent years. Given the opposing roles that have been reported for eosinophils in cancer[9-18], it is likely that phenotypic and functional diversity in the eosinophil population exists, a concept that is now widely accepted for other tumor-infiltrating myeloid populations such as macrophages and neutrophils[39,40].

The concept of eosinophil heterogeneity has been explored in the context of inflammatory diseases[41]. In a mouse model of allergic airway inflammation, two distinct sub-populations of eosinophils with opposing functions were described: *resident/regulatory* eosinophils (defined as: SiglecF^{int} CD62L^{+} CD101^{-}) and *inflammatory* eosinophils (defined as: SiglecF^{high} CD62L^{-} CD101^{+})[42]. RNA sequencing analysis and mechanistic studies revealed that the *resident* eosinophils were found in homeostatic as well as allergic lungs and had a regulatory function via inhibition of allergen loaded DCs. In contrast, the population of *inflammatory* eosinophils was only detected in allergic airway inflammation and promoted Th2-related inflammation in the lungs[42]. These findings were also relevant in humans, where the authors identified distinct eosinophil subsets in lung tissue of healthy individuals and the sputa of patients with eosinophilic asthma based on differential surface receptor expression of Siglec8, CD62L and IL-3R[42]. Others have categorized eosinophil heterogeneity as *progenitors, steady state eosinophils,* and *type 1* and *type 2 activated phenotypes*, based on eosinophil diversity shaped by different tissue microenvironments during homeostasis and disease[43]. Most recently, single-cell transcriptomic profiling identified five eosinophil subpopulations - *eosinophil precursors, immature, circulating, basal,* and *active eosinophils* - present in various mouse tissues upon colitis[44]. Whether these eosinophil phenotypes originate from different eosinophil subpopulations of distinct developmental origin or reflect a continuum of activation states, remains a matter of contention.

Despite these advances in our understanding of eosinophil heterogeneity in inflammatory diseases, our knowledge of eosinophil phenotypes in cancer remains scarce. The existence of both *resident* and *inflammatory* eosinophil phenotypes, based on SiglecF, CD62L and CD101 expression as identified in the allergic airway inflammation model[42], was described in the lungs of mice harboring 4T1 breast cancer-derived metastases[18]. Comparison of the transcriptional profile of SiglecF^{high} and SiglecF^{int} eosinophils sorted from the metastasis-bearing lungs revealed transcriptional convergence of these two populations in comparison to circulating eosinophils[18], arguing that the phenotype of metastasis-infiltrating eosinophils is predominantly instructed by the tumor. Our explorative RNA sequencing analysis suggested that transcriptional diversity exists in circulating eosinophils sorted from mice treated with CIS + ICB compared to control antibody (**chapter 3**). This observation raises the question whether immunotherapy causes the accumulation of eosinophils with a specific activation state or whether immunotherapy selectively expands a particular subpopulation of eosinophils (**Figure 2A**). To address this question, we further investigated eosinophil phenotypes upon CIS + ICB therapy by flow cytometry. Comparing eosinophils in the circulation of KEP tumor-bearing mice to tumor-free wild-type mice, we observed an increased frequency of SiglecF^{high} eosinophils in tumor-bearing mice, which was further enhanced upon treatment with CIS + ICB (**Figure 2B**). SiglecF^{high} eosinophils expressed higher levels of CD101 and lower CD62L compared to their SiglecF^{int} counterparts (data not shown), in line with the previously described phenotype of *inflammatory* eosinophils[42]. Although CIS + ICB increased the proportion of SiglecF^{high} eosinophils on total eosinophils, it also promoted the accumulation of SiglecF^{int} eosinophils when measured as frequency of total immune cells (data not shown). In KEP mammary tumors, only one population of eosinophils could be identified based on SiglecF expression. However, whereas eosinophils in tumor-free mammary glands were characterized as CD62L^{-} CD101^{-}, the predominant phenotype of tumor-infiltrating eosinophils was CD62L^{-} CD101^{+} (**Figure 2C**), reminiscent of the *inflammatory* eosinophil phenotype described in the asthmatic lung[42]. Interestingly, also in our eosinophil engagement study using combined ICB + rIL-33 treatment, we found increased frequency of SiglecF^{high} eosinophils in the circulation compared to control, whereas eosinophils infiltrating the tumors were predominantly CD62L^{-} CD101^{+} (*inflammatory*), regardless of treatment (**Figure 2D, E**). Whether eosinophils acquire this so-called *inflammatory* phenotype upon entering the tumor or whether *inflammatory* eosinophils are preferentially recruited to the tumor remains to be investigated (**Figure 2A**).

Our inability to selectively deplete one of these eosinophil sub-populations/activation states, hampers our mechanistic understanding of their functional significance. However, we hypothesize that these phenotypic alterations are important for the anti-tumoral function of eosinophils upon immunotherapy. Evidence to support this notion comes from an intriguing study by *Carretero* and colleagues. They found that the effectiveness of adoptive T cell transfer of OT-I T cells in MO4 melanoma model relied on co-transfer with IFNγ+TNFα-activated eosinophils, but co-transfer with non-activated eosinophils was ineffective, demonstrating that eosinophil activation state is essential for their function. Our explorative RNA sequencing analysis also suggested that CIS + ICB combination

therapy promotes transcriptional changes indicative of IFNγ-induced activation. IFNγ is a known activator of eosinophils in different inflammatory diseases[45] and was shown to directly enhance the cytotoxicity of eosinophils towards colorectal cancer cells[15]. Similarly, IL-33 has been shown to activate eosinophils, promoting eosinophil cytotoxicity of tumor cells via enhanced degranulation[14]. Recently, it was shown that combined IL-33 and IFNγ induced accumulation of activated eosinophils with bactericidal and T cell regulatory functions in the inflamed colon[44]. It will be important to further characterize eosinophils at a molecular and phenotypic level in clinically relevant mouse models and cancer patients upon ICB treatment to evaluate whether a specific activation state can be linked to ICB response.

How might we tackle this question? Eosinophils are notoriously difficult to analyze using RNA sequencing techniques due to their low RNA content and high expression of RNAses, which are detrimental for RNA quality, complicating the phenotypic assessment of eosinophils. As eosinophil research in cancer is gaining popularity, development of new techniques and increased sensitivity of current sequencing methods are expected to provide more insights. This is exemplified by the recent paper of *Gurtner* and colleagues describing a pipeline for successful single-cell RNA sequencing of mouse eosinophils in a colitis model. This involved the use IL-5-overexpressing mice to increase eosinophil numbers, the pooling of samples of 3-4 mice per condition, and using an anti-SiglecF based bead-isolation instead of fluorescence-activated cell sorting strategy for eosinophil isolation to reduce shear stress and prevent consequent degranulation, release of RNAses and transcript degradation[44]. Whether this strategy is sufficient to isolate a pure population of eosinophils from tumors remains to be tested as subsets of intratumoral neutrophils express SiglecF as well[46]. Alternatively, other techniques such as proteomics may be more practically feasible and may shed more light on the functional state of this elusive cell population in cancer.

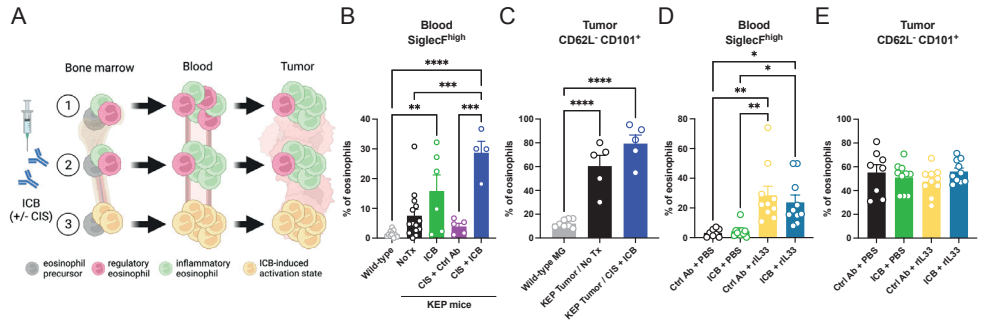


Figure 2. Assessment of eosinophil phenotypes upon immunotherapeutic strategies
(A) Schematic representation of how ICB (+/- CIS) may affect eosinophil phenotype in the circulation and tumor. ICB (+/- CIS) may lead to (1) the expansion of both *regulatory* and *inflammatory* eosinophils subsets of distinct developmental origin, but only *inflammatory* eosinophils are recruited to the tumor, (2) the selective expansion of *inflammatory* eosinophils, leading to their enhanced recruitment to the tumor, or (3) the expansion and tumor recruitment of eosinophils in a particular ICB-induced activation state. (B) Frequency of SiglecF^{high} eosinophils in the blood of tumor-free wild-type or tumor-bearing KEP mice treated as indicated, analyzed by flow cytometry at tumor-related endpoint (n=5-14). (C) Frequency of CD62L^{-} CD101^{+} eosinophils infiltrating the mammary gland of tumor-free wild-type or mammary tumors of KEP mice treated as indicated, analyzed by flow cytometry at tumor-related endpoint (n=5-7).

Legend continues on the next page

(D) Frequency of SiglecF^{high} eosinophils in the blood of mice bearing orthotopically transplanted KEP tumors, treated as indicated, analyzed by flow cytometry at tumor-related endpoint (n=8-10). (E) Frequency of CD62L^{-} CD101^{+} eosinophils infiltrating in orthotopically transplanted KEP tumors of mice treated as indicated, analyzed by flow cytometry at tumor-related endpoint (n=8-10). All data are mean ±S.E.M., statistical analysis by One-way ANOVA with Tukey's correction. ns, not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Eosinophil engagement to improve immunotherapy efficacy

We uncovered a mechanism by which ICB-activated IL-5-producing CD4^{+} T cells instigate eosinophil development in the bone marrow leading to the systemic expansion of eosinophils. Additional induction of IL-33 upon combined cisplatin and ICB triggered intratumoral eosinophil infiltration, where eosinophils contribute to anti-tumor immune response by promoting CD8^{+} T cell activation. We proposed that engagement of eosinophils may enhance therapeutic efficacy of immunotherapy in absence of chemotherapy. The correlation we observed between IL-33 expression and the presence of an eosinophil signature in metastatic lesions of breast cancer patients who respond to ICB, combined with our experimental study demonstrating that the effectiveness of ICB can be enhanced by inducing eosinophil mobilization through systemic administration of recombinant IL-33, indicates that IL-33 could be an attractive engager of eosinophils in breast cancer patients upon ICB. Nevertheless, the systemic administration of rIL-33 as performed in **chapter 3** induced only a modest therapeutic benefit over ICB, leaving space for improving the use of rIL-33 and the assessment of additional eosinophil engagement strategies. Moreover, as outlined above, it will be critical to assess whether these engagement strategies induce the 'right' kind of eosinophil activation to contribute to anti-tumor immunity.

IL-33 is an alarmin, best known for its function to amplify the inflammatory response by recruiting eosinophils and other type 2 immune cells to sites of inflammation and tissue damage[47]. IL-33 is involved in eosinophil recruitment as well as activation[14]. However, when contemplating the use of IL-33 for eosinophil engagement, one must consider the pleiotropic functions of IL-33[48], some of which may counteract its beneficial effect on eosinophils. Importantly, IL-33 has been shown to instruct the expansion and immunosuppressive function of T_{regs}, thereby contributing to cancer progression[49,50]. Promisingly, previous studies in our lab have shown that T_{reg} accumulation in KEP mice is independent of IL-33[51] and we observed no differences in T_{reg} expansion or activation upon systemic recombinant IL-33 delivery (**chapter 3**). In a model of gut helminth infection, it was demonstrated that the cellular context in which IL-33 is expressed matters for its activity[52]. Broad release of IL-33 from damaged epithelial cells was linked to ILC2-meditated helminth clearance, while dendritic cell-derived IL-33 induced immunosuppressive T_{regs} and impaired worm clearance. Careful assessment of the cellular source of IL-33 upon immunotherapy in mouse models as well as breast cancer patient samples may provide further clues how to optimally deliver IL-33 during immunotherapy, for instance via intratumoral injections.

Interestingly, the use of IL-33 to induce anti-tumor immunity is gaining popularity for reasons beyond its potential effect on eosinophils. It has been reported that IL-33 directly affects CD8^{+} T cells, promoting their expansion and maintenance of stemness phenotype during chronic viral infections[53]. Moreover,

treatment with aCTLA-4 or aPD-1 was demonstrated to induce IL-33 expression in MC-38 colon cancer cells which enhanced the accumulation and effector functions of tumor CD8$^+$ T cells and DCs, which was required for the anti-tumor efficacy of ICB[54]. Adoptive transfer of OT-I T cells engineered to secrete an IL-2 variant and IL-33 provoked a T cell state with superior effector functions, leading to tumor regression of B16-OVA tumors[55]. The secretion of IL-33 was critical, but its effects were shown to be T cell extrinsic, thus likely mediated through reprogramming of the TME. Unfortunately, the authors did not assess which components of the TME may be responsible. Similarly, co-expression of IL-2 superkine and IL-33 in CAR T cells improved anti-tumor responses in multiple solid tumor models, via the induction of broad innate and adaptive immune cell activation in the TME[56]. In our study in **chapter 3**, we cannot exclude additional roles for IL-33 beyond eosinophil engagement, such as direct CD8$^+$ T cell or DC activation upon CIS + ICB. Nevertheless, our data demonstrate that IL-33 is an attractive approach to engage eosinophils and enhance immunotherapy efficacy, but further studies are needed to evaluate whether IL-33 in combination with ICB can be used to engage eosinophils safely and specifically in cancer patients.

Which other strategies may be used to engage eosinophils to improve ICB responses? An attractive approach is the inhibition of dipeptidyl peptidase 4 (DPP4). DDP4 is a ubiquitously expressed serine protease involved in the truncation and degradation of certain chemokines, thereby regulating their biological activity. DDP4 inhibitors are FDA-approved for the treatment of diabetes type 2 and work via blocking the degradation of insulinotropic hormones such as GLP-1[57]. However, among DPP4's targets is also CCL11, an eotaxin that is essential for eosinophil trafficking to sites of inflammation via engagement of its receptor CCR3 expressed on eosinophils[58]. Blockade of DPP4 was shown to increase the recruitment of eosinophils in a CCL11-CCR3 dependent manner in a rat skin inflammation model[57]. In the hepatocellular carcinoma and breast cancer models, DDP4 inhibition not only stabilized CCL11 expression in the tumor, but also induced an increase in intratumoral IL-33 levels. These changes led to increased eosinophil infiltration in the tumor and IL-33-dependent eosinophil-mediated tumor cell cytotoxicity[14]. DDP4 inhibitors are approved for the treatment of type 2 diabetes mellitus with good safety profiles[59], which could accelerate their clinical implementation for cancer patients. However, it is crucial to evaluate the expression pattern of DPP4 in tumor tissues of cancer patients as well as consider the potential off-target effects on other chemokines involved in cancer.

Given the essential role for eosinophils in fighting parasites and other extracellular pathogens[6], it is enticing to consider whether eosinophils can be engaged to combat cancer with parasite-derived molecules. Interestingly, with improved control of parasitic infections in developed countries, came a simultaneous increase in the incidence and mortality of cancer[60]. This raised the intriguing hypothesis that parasitic infections may somehow interfere with cancers, either by directly inhibiting tumor growth or by educating the immune system in favor of anti-tumor immunity[61]. Experimental studies have demonstrated the potential anticancer properties of certain parasites, including *Trypanosoma cruzi, Toxoplasma gondii, Toxocara canis, Acantamoeba castellani,* and *Plasmodium yoelii*[61]. The mechanisms

underlying the tumor resistance induced by parasites are not fully understood, however, it is believed that various mechanisms, including the presentation of common antigens and activation of innate or acquired immunity may be involved. Some parasites are known to produce mucin-type O-glycans that are also present in cancer cells, indicating the existence of shared antigens between parasites and cancers[62]. It has been suggested that *Trichinella spiralis* infection enhances anti-tumor immunity via induction of eosinophils capable of direct tumoricidal activity[63]. Whether this strategy can be employed in combination with ICB remains to be investigated. Similarly, treatment with fungal-derived β-glucans has been shown to enhance anti-tumor immunity via activation of inflammatory monocytes[64] or reprogramming of neutrophils[65], but whether β-glucans affect eosinophils and can enhance immunotherapy efficacy is currently unknown. While the idea that parasite-derived agents may have potential in engaging eosinophils to improve cancer immunotherapy outcome is fascinating, more research is needed to understand the mechanisms involved, and to determine the safety and efficacy of these treatments in patients.

Lastly, our studies concerning the adverse role of T_{regs} (**chapter 4**) and neutrophils (**chapter 5**) in cancer immunotherapy response revealed remarkable interconnectivity between these immunosuppressive cell types and eosinophils. Short-term neutrophil depletion using anti-Ly6G during ICB therapy in KEP mice induced a relative increase in eosinophils in the circulation. More strikingly, T_{reg}-depletion, independent from ICB therapy, induced massive eosinophil infiltration into the tumor. These data indicate that targeting the immune suppressive network might be a viable strategy to engage eosinophils and promote $CD8^+$ T cell activation concurrently. Whether the eosinophil accumulation observed upon T_{reg}-depletion in KEP mice is similarly driven by the IL-5-IL-33-axis and whether these eosinophils are activated into an anti-tumorigenic state, is currently unknown. Investigation of cytokine and chemokine expression in tumor lysates isolated from mice treated with ICB + T_{reg}-targeting may reveal additional factors that drive eosinophil recruitment to the tumor that can be exploited therapeutically.

Eosinophils, allergies, and immunotherapy response

Allergic reactions stem from hyperactivity of the immune system, whereas cancers induce a chronic inflammatory, yet immunosuppressed state. As such, the relationship between cancer and allergy has long since fascinated researchers. Although cancer prevalence in general is not clearly linked to a history of allergy, some intriguing relationships have been found for subtypes of cancer[66]. For instance, the risk of pancreatic, colon and brain cancer is reduced among allergic patients, regardless of the type of allergy they have[67-69]. These observations suggest that allergies enhance systemic immunosurveillance, protecting certain tissues from cancer incidence. In other organs however, allergies have been shown to contribute to inflammation-induced carcinogenesis, which is particularly apparent in tissues exposed to many carcinogens as well as allergens such as the lungs[66,68,70].

Eosinophils play a central role in the pathophysiology of certain allergies[6]. Conceptually, this raises some intriguing questions regarding the role of eosinophils in allergic responses and cancer. In the current age of immunotherapy for cancer, a

new question arises: how may allergies affect immunotherapy efficacy? Given our findings in **chapter 3** that eosinophils are essential for ICB responses and induced via an IL-5-dependent manner similar as during allergic inflammation[26,27], it is tempting to speculate that eosinophilic allergies may enhance anti-tumor immune responses. There is some evidence to suggest this may be the case. In addition to their increased frequency during allergies, eosinophils have been shown to have increased cytotoxic ability under allergic conditions[71] and eosinophils from allergic donors showed enhanced cytotoxicity towards colorectal cancer cells compared to those of healthy donors[72]. However, most of these studies are based on *in vitro* experiments and the contributions of other allergic mediators such as mast cells were not considered.

Evaluation of the associations between eosinophils, allergy and cancer immunotherapy response is complex as assessment of the allergic status of cancer patients undergoing immunotherapy oftentimes relies on self-reporting and apparent allergic symptoms may be confounded by symptoms of other diseases[66]. Moreover, the widespread use of anti-inflammatory drugs to mitigate allergic symptoms may be a confounder too, as these drugs can have implications for immunotherapy efficacy in unexpected ways. For instance, a retrospective analysis demonstrated that patients who took antihistamines during immunotherapy for the treatment of metastatic melanoma or lung cancer had improved survival[73]. Mechanistically, this was mediated via histamine expression by cancer cells and histamine receptor H1 expression on TAMs, which induced their M2-like pro-tumorigenic polarization, which in turn caused T cell dysfunction in the TME. To assess the relationship between eosinophils, allergy and immunotherapy response, more research is needed on the molecular level in *in vivo* tumor models as well as cancer patients. These studies should take into account the potential organ-specific differences outlined above and carefully consider the role of other mediators of allergies such as mast cells and neutrophils, which may have opposing functions in immunotherapy response compared to eosinophils.

Simultaneous boosting of anti-tumor immunity and dampening tumor-promoting inflammation and immunosuppression

In this thesis I argue that simultaneous boosting of anti-tumor immunity and dampening of tumor-promoting inflammation and immunosuppression can improve responses to immunotherapy, circumventing the need for chemotherapy. This concept was explored in **chapter 4** and **5**.

Immunosuppression in the TME comes in many shapes and forms, ranging from cellular components to soluble mediators and physiological properties of tumors. Expression of T cell inhibitory ligands such as PD-L1[74], production of immunosuppressive cytokines such as IL-10 and TGFβ[75,76], consumption of essential nutrients[77,78], induction of hypoxia[79] and release of reactive oxygen species[80] are among the many immunosuppressive mechanisms in the TME. These immunosuppressive mechanisms are employed by cancer cells themselves, but equally, if not more important, are the contributions of tumor-infiltrating immune cells[81]. For instance, PD-L1 expression on tumor-infiltrating myeloid cells was shown to be a more significant determinant for anti-PD-L1 therapy-mediated tumor

regression than PD-L1 expression on cancer cells[74], highlighting the dominant role of myeloid-derived immunosuppression in the TME. A major contributor to immunosuppression in the TME are TAMs, which have been extensively studied by our group and others[82-84]. In this thesis, we focused on two other key orchestrators of tumor-induced immunosuppression: regulatory T cells[85] (**chapter 4**) and neutrophils[86] (**chapter 5**).

Tumor-induced inflammation and immunosuppression reaches far beyond the TME. This is particularly evident for neutrophils, whose increased development in the bone marrow and consequent systemic expansion and immunosuppressive polarization have been particularly implicated in metastasis formation[87-93]. Conversely, the role of T_{regs} in cancer and immunotherapy response has been predominantly studied in the primary tumor setting in models intrinsically sensitive to ICB[94-99]. Our lab has previously shown that KEP tumors drive the systemic expansion of activated, immunosuppressive T_{regs} that promote metastatic spread to the lymph nodes, but not the lung, through local suppression of NK cells[100]. Our data in **chapter 4** confirmed this finding as none of the mice treated with T_{reg}-depletion developed lymph node metastasis. Others have suggested that neutrophils are more immunosuppressive to T and NK cells in the lungs compared to other organs, driving breast cancer metastasis to the lungs[92]. These observations emphasize that organ specific differences in the immunosuppressive network exist, which have consequences for metastatic spread and response to immunotherapy. Reversely, the location of metastases may also impact response to immunotherapy. For instance, presence of liver metastasis has been correlated to reduced response to PD-1/PD-L1 blockade in metastatic TNBC patients[101,102]. In mouse models, it was shown that liver metastasis-associated macrophages eliminate activated $CD8^+$ T cells via apoptosis induction, reducing immunotherapy efficacy[103].

Appreciation for the importance of systemic immunity is gaining traction in the immunotherapy field[104]. Indeed, in **chapter 3** and **6** we demonstrate that effective anti-tumor response to combined cisplatin + aPD-1/CTLA-4 or cisplatin + muPD1-IL2v was characterized by not only intratumoral immune activation but also systemic activation in the blood and tumor-draining lymph nodes. In cancer patients, it was shown that surgical removal of the regional lymph nodes negatively correlates with response to ICB[105]. In mouse models, resection of the tumor-draining lymph nodes prevented immunotherapy-induced tumor control by decreasing immune cell infiltration in the tumor[106]. Another study showed that upon immunotherapy, initial immune activation was observed both within the tumor and throughout peripheral tissues. However, during the process of tumor rejection, only immune cells in the periphery continued to undergo cell division. This coordinated response across different tissues was necessary for the eradication of tumors in several immunotherapy models, underscoring the importance of systemic immunity in mounting effective immunotherapy responses[107]. Thus, to further improve our understanding of the requirements of immunotherapy efficacy, we must take a holistic approach by assessing the systemic immune landscape as well as the tumor microenvironment, keeping potential organ-specific differences in the immunosuppressive network in mind.

Regulatory T cells; the puppet master of the immunosuppressed TME

T_{regs} are essential safe guardians of immune homeostasis, preventing autoimmunity and excessive responses to pathogens[108]. T_{regs} can make up a substantial proportion of tumor-infiltrating lymphocytes depending on the cancer type[109]. In addition, increased ratio of T_{regs} versus effector T cells in tumor tissue has been correlated to worse clinical outcome in many solid cancer types[110]. Interestingly, correlations between T_{regs} and prognosis in breast cancer patients seem to vary per breast cancer subtype. In hormone receptor (HR)-positive breast cancer, high intratumoral T_{regs} have been correlated to poor prognosis[111], whereas T_{regs} have been correlated to favorable prognosis in HR-negative breast cancer and TNBC[85]. In TNBC, increased T_{regs} in the tumor was strongly correlated with increased infiltration of other lymphocytes including $CD8^+$ and $CD4^+$ T cells[112,113], suggesting that the association of T_{regs} with favorable prognosis is more reflective of a general increase in T cell infiltration in TNBC. This breast cancer subtype has a higher mutational burden, more T cell inflamed TME, and shows the highest response rate to ICB[114-116].

It is important to realize that T_{regs} may be direct targets for ICB therapy, as the expression of immune checkpoint molecules such as PD-1 and CTLA-4 is not limited to $CD8^+$ T cells but is also found on intratumoral T_{regs}[117-119]. Indeed, in **chapter 4** we found that aPD-1/CTLA-4 therapy inadvertently activates and expands immunosuppressive T_{regs} in mammary tumor models. Also in breast cancer patients, we found indications that T_{reg} levels are elevated upon ICB therapy in the tumor and circulation. These observations are in line with previous studies showing that both anti-PD-1 and anti-CTLA-4-based antibody therapies can induce the activation and proliferation of T_{regs}[120-122] and are consistent with clinical observations that expression balance of PD-1 on intratumoral T_{regs} over $CD8^+$ T cells correlates with non-responsiveness to PD-1 therapy in patients with melanoma, NSCLC, and gastric cancer[119]. We also found that the expression of PD-1 and CTLA-4 was highest on tumor T_{regs} across all breast cancer models currently used in the lab (**chapter 4** and data not shown). Nevertheless, not all models were resistant to ICB therapy, suggesting that simply determining the expression balance of PD-1 on T_{regs} over $CD8^+$ T cells may be insufficient to predict ICB responses in breast cancer. Other factors including differences in T cell infiltration, myeloid cell infiltration and PD-L1 expression on myeloid cells, are expected to contribute as well. Interestingly, in our studies **chapter 6** we observed hyperprogression of KEP tumors upon aPD-1 therapy using the Fc-modified anti-PD-1 antibody we used as a control reagent for muPD1-IL2v obtained from Roche. Hyperprogression upon aPD-1 therapy is observed quite frequently in cancer patients[123,124] and was linked to expansion of $PD\text{-}1^+$ T_{regs} in gastric cancer patients[123]. As studying the efficacy of this PD-1-targeting antibody was not the primary interest of our study, we did not perform any follow-up experiments on this finding. However, it would be interesting to assess in more detail whether activation of $PD\text{-}1^+$ T_{regs} may have contributed to the hyperprogression we observed.

Moreover, it is currently unclear whether T_{regs} are directly stimulated by aPD-1/aCTLA-4 therapy, resulting in increased proliferation and immunosuppressive function[123,125], or whether the T_{reg} expansion observed upon ICB therapy is a result of the upregulation of immunoregulatory feedback mechanisms following

an ongoing $CD8^+$ T cell response[122]. Nevertheless, investigating the molecular mechanisms underlying the induction of T_{regs} by ICB could provide valuable insights for the development of immunotherapeutic approaches that specifically activate conventional T cells, but not T_{regs}. Besides PD-1 and CTLA-4, T_{regs} have been shown to express other immune checkpoints for which agonistic or antagonistic antibodies are in clinical development including TIGIT, LAG3, TIM3, and 4-1BB[126-129]. The expression levels of immune checkpoints on T_{regs} versus conventional $CD4^+$ and $CD8^+$ T cells should be taken into account when treating patients with immune checkpoint inhibitors and considered during the development of new immunomodulatory drugs, particularly in patients with high T_{reg} infiltration in the tumor. Efforts to selectively activate $CD8^+$ T cells over T_{regs} should be made. We investigate one such approach in **chapter 6**, which will be discussed in more detail in a paragraph below.

The ICB-induced accumulation and activation of T_{regs} we observed in mammary tumor-bearing mice, affected both intratumoral and systemic immunosuppression. The potency of T_{regs} in controlling the intratumoral immune landscape was quite astonishing. Short-term T_{reg}-depletion during neoadjuvant ICB caused a drastic re-wiring of the TME with increased $CD8^+$ T cells, $CD4^+$ T cells, and eosinophils, while decreasing conventional dendritic cells type 2 (cDC2s) and neutrophils. In addition, we observed strong upregulation of MHC-II on inflammatory monocytes and TAMs. A recent study in *Kras*$^{LSL\text{-}G12D/WT}$*;Trp53*$^{F/F}$ lung adenocarcinoma model found similar interconnectivities between T_{regs} and many other tumor-infiltrating cells. Responses to DT-mediated T_{reg}-depletion became apparent in fibroblasts, endothelial cells and myeloid cells as early as 48 hours after T_{reg}-depletion, before any notable changes in T cell activation could be observed[130]. It is currently unclear what the exact interconnections are between T_{regs} and all these cell populations. Do T_{regs} suppress these populations directly or does the lifting of immunosuppressive network induce a cascade of events, leading indirectly to reprogramming of several other immune parameters associated with anti-tumor immunity?

Unlike previous studies involving tumor cell lines with higher immunogenicity[94-97], we observed no therapeutic benefit of T_{reg}-targeting alone. Moreover, the anti-tumoral effects of combined ICB + T_{reg}-depletion were only evident in the metastatic context, not in primary tumors. This suggests the existence of additional obstacles to anti-tumor immunity in primary KEP tumors that are unrelated to T_{regs}. A likely explanation is the remaining presence of other immunosuppressive cell types such as macrophages[131]. Previous studies have highlighted the reciprocal interactions between T_{regs} and TAMs[132,133], emphasizing the crosstalk between these two immunosuppressive subsets and the existence of compensatory mechanisms driving resistance to immunotherapies. Another possible explanation lies in the broad upregulation of PD-L1 on intratumoral myeloid cells we observed upon T_{reg}-depletion, indicative of negative feedback mechanisms in place to limit immune activation. Although PD-L1 positivity at baseline is associated with response to ICB strategies[74], upregulation of PD-L1 during ICB therapy is also described as an acquired resistance mechanism limiting therapy efficacy[5]. Upregulation for PD-L1 in the tumor has local effects limiting anti-tumor immunity but can also protect distal metastases from systemic immunity[107].

Despite being insufficient to control primary tumor growth, we hypothesized that the broad pro-inflammatory changes in the TME induced by T_{reg}-depletion, may have contributed to the development of a robust anti-metastatic immune response in combination with ICB. Indeed, combined ICB and T_{reg}-depletion induced a systemic increase in $CD8^+$ T cells, $CD4^+CD8^+$ T cells, NK cells, and eosinophils. Moreover, combination therapy led to durable $CD8^+$ T cell activation, and increased $CD8^+$ T cell counts were detectable in metastatic lesions more than 7 weeks after cessation of neoadjuvant therapy. We have investigated the cellular dependency of therapeutic benefit of ICB + T_{reg}-depletion on $CD8^+$ T cells and NK cells. Whereas NK cell-depletion had no effect, we demonstrated that $CD8^+$ T cells are the main, but not sole driver of anti-metastatic response. As discussed above, T_{reg}-depletion also induced systemic expansion and tumor infiltration of eosinophils. Would we lose the anti-metastatic effect of ICB and T_{reg}-targeting upon eosinophil depletion? Moreover, the functional role of $CD4^+$ T cells in the anti-tumor immune response upon ICB and T_{reg}-depletion could be considered more closely. Upon neoadjuvant ICB and T_{reg}-depletion, we observed a decrease in cDC2s in the tumor as well as strong increase in $CD4^+$ T cell accumulation and activation. These findings are in line with a previous study that showed how T_{reg}-depletion induces the migration of cDC2s from the tumor to the TDLNs, where they gained increased ability to prime and activate $CD4^+$ T cells in absence of T_{regs}, leading to $CD4^+$ T cell-mediated rejection of the B16 melanoma tumors[134]. As we kept the mice alive after mastectomy to monitor metastasis formation, we could not analyse the TDLNs, and thus we do not know whether the observed decrease in tumor cDC2 levels is due to increased migration to the TDLN in our model. Assessing whether $CD4^+$ T cells may play a role in the observed anti-metastatic response would be of interest for future studies.

From our data in **chapter 4** and that of others, it is clear that T_{regs} play a central role in limiting anti-tumor immunity and immunotherapy responses, making them attractive targets for improving the efficacy of immunotherapy. However, it is important to maintain the balance between anti-tumor immunity and prevention of autoimmune reactions and immune-related adverse events. The major challenge in advancing T_{reg}-targeting to clinical implementation is to safely target T_{regs} in cancer patients. Efforts should focus on approaches that specifically deplete intratumoral T_{regs} or that only partially deplete T_{regs}. Several approaches are being investigated, including the repurposing of existing drugs such as gemcetibine[135] and discovering new targets like OX-40, CD25, and CCR4[95,108,123]. A highly promising target is the chemokine receptor CCR8. CCR8 expression is predominantly found on a highly immunosuppressive subset of intratumoral T_{regs} across various cancer types[136], including breast cancer patients[137]. Preclinical studies using cancer cell line inoculation models including EMT6 and 4T1 breast cancer models, have demonstrated that anti-CCR8 therapy can selectively reduce intratumoral T_{regs}, leading to tumor control as well as improved responses to aPD-1 therapy, with minimal autoimmune-related side effects[97,138,139]. Whether anti-CCR8 can enhance responses to ICB in spontaneous cancer models, such as the KEP model, which better simulate the inflamed and immunosuppressed conditions observed in cancer patients, remains to be investigated. Currently, anti-CCR8 antibodies are being tested in early-phase clinical trials. Future research should continue to explore strategies to safely

target T_{regs} in cancer patients, with the ultimate goal of improving the efficacy of immunotherapy while reducing the incidence of immune-related adverse events.

Neutrophils; a formidable opponent of dynamic nature

Despite gaining increasing attention in recent years, controversy regarding the role of neutrophils in immunotherapy response remains. This controversy is likely related to the neutrophil heterogeneity and plasticity in phenotype and function, which appears to depend on tumor type, tumor stage and type of immunotherapy that is used. Our lack of understanding the impact of neutrophils on cancer immunotherapy also sprouts from the limited tools for targeting neutrophils efficiently[140,141]. This, in turn, relates back to the highly dynamic and plastic nature of this abundant myeloid immune cell population. It is estimated that 10^7 neutrophils are produced in mice per day, and in humans this number increases to a staggering 10^{11} neutrophils daily. During cancer progression, granulopoiesis is pushed to its limits, leading to the release of more immature neutrophils with altered phenotypes and functions. Perhaps is it not surprising that depleting neutrophils against this flood of new production is like shoveling sand against the tide. Previous research in our lab indicates a clear pro-tumorigenic and immunosuppressive function for neutrophils in KEP mammary tumors and metastases[87,88]. Our data of **chapter 5** demonstrate that neutrophils also counteract immunotherapy efficacy by suppressing $CD8^+$ T cell activation in the TME, highlighting that neutrophil modulating strategies may improve responses to immunotherapeutic strategies in breast cancer patients. How might we safely and efficiently target such a dynamic myeloid population in cancer patients?

Depleting neutrophils from cancer patients could increase their susceptibility to severe infections[142], therefore approaches that prevent neutrophil recruitment to the TME are under intensive investigation. Targeting of CXCR2 has been shown to inhibit cancer-induced neutrophilia and improve responses to immunotherapy in preclinical cancer models[143,144]. However, not all neutrophils express CXCR2 or rely on CXCR2 for their recruitment. For instance, immature neutrophils, which are abundant in preclinical tumor models and cancer patients[87,145], express lower levels of CXCR2 than fully mature neutrophils[146]. Moreover, the long-term effects of manipulating the recruitment of a dynamic myeloid population need to be carefully evaluated. For instance, retention of monocytes in the bone marrow by CCL2 neutralization inhibited metastasis formation in spontaneous breast and pancreatic cancer models[147,148]. However, cessation of anti-CCL2 treatment in patients caused a compensatory influx of monocytes into metastatic sites and resulted in increased mortality[149]. Additionally, compensatory mechanisms may exist between the different arms of the immunosuppressive network. Targeting one immunosuppressive cell type may lead to the recruitment of another that takes over its function[84,150]. Indeed, a compensatory influx of neutrophils was observed after TAM depletion in transplantable mouse models of melanoma, lung, colon and breast cancer[151,152]. CSF-1R inhibition was found to have an unexpected pro-metastatic effect by indirectly reducing NK cell numbers via removal of TAM derived IL-15 survival signal for NK cells[153]. These studies underscore how challenging it can be to target the dynamic interactions between cancer cells and immune cells,

particularly within the myeloid compartment.

Another complicating factor is the observation that neutrophils, or particular subsets of neutrophils, may contribute to anti-tumor immunity and immunotherapy responses in certain instances. Although the bulk of experimental evidence points towards pro-tumorigenic and immune suppressive role for neutrophils[154,155], some studies have shown that neutrophils can contribute to anti-tumor immunity[156,157]. A recent study in the orthotopically transplanted KP lung adenocarcinoma model revealed that successful immunotherapy using a CD40 agonist resulted in an increase in neutrophil infiltration in the tumor, which contributed to tumor control[157]. This occurred through the selective expansion of the $CD62L^{high}$ $SiglecF^{low}$ neutrophil subset, which had anti-tumorigenic properties, while the immunosuppressive $SiglecF^{high}$ neutrophil subset remained unchanged. However, aPD1/CTLA-4 therapy, which did not provide therapeutic benefit in the KP model, did not result in the same phenomenon. This is in line with our own findings in mice bearing KEP tumors or metastases, which showed that neutrophil infiltration and SiglecF expression were unaffected by unsuccessful aPD-1/aCTLA-4 therapy (**chapter 5**). These results suggest that neutrophils can have different effects on immunotherapy response depending on the type of checkpoint inhibitors used and whether an initial anti-tumor T cell response is achieved.

Instead of targeting the entire neutrophil population, efforts to reprogram neutrophils from a pro-tumorigenic to anti-tumorigenic state is a preferred strategy. We have previously identified several of the upstream mediators that induce neutrophil expansion and polarization in KEP mice, including G-CSF, IL-17, and IL-1β[87]. The cancer field can make use of antagonists that are in clinical development for inflammatory diseases, some of which are indeed already FDA approved. Combining ICB with anti-IL-1β is of particular interest as we have previously shown that IL-1β production by tumor-infiltration macrophages is an early event in the cascade promoting the emergence of immunosuppressive neutrophils in KEP mice[87,158]. Anti-IL-1β treatment normalized the level and phenotype of neutrophils in primary tumor-bearing KEP mice[87]. Our preliminary data suggest that addition of anti-IL-1β to neoadjuvant + adjuvant ICB therapy may improve anti-metastatic responses when compared to ICB monotherapy, despite having only minimal effect on reducing circulating neutrophil levels (**Figure 3**). We are currently repeating this experiment with an independent tumor donor and higher dosing frequency of anti-IL-1β to strengthen these findings.

Interest in anti-IL-1β as a cancer therapeutic was sparked by a serendipitous finding. A retrospective analysis of a phase III cardiovascular study using anti-IL-1β showed significantly reduced lung cancer incidence[159]. This observation spurred several clinical trials to evaluate the safety and tolerability of combining anti-IL-1β with aPD-1 in lung cancer patients. Although the first press releases of these CANOPY-studies stated that the trial did not meet its primary endpoints, the data suggest that certain subgroups of patients may benefit from this treatment combination. Unfortunately, no data has been released on neutrophil frequencies or related mediators from these trials. Identifying which cancer patient subgroups are expected to benefit from neutrophil-targeting strategies will be important going forward. Moreover, it will be important to monitor neutrophil levels during anti-

IL-1β + aPD-1 therapy in these patients, as a drawback of utilizing a therapy that blocks a single cytokine is that the effectiveness of the treatment may be reduced due to functional redundancy among cytokines or compensatory effects from other cytokines[160].

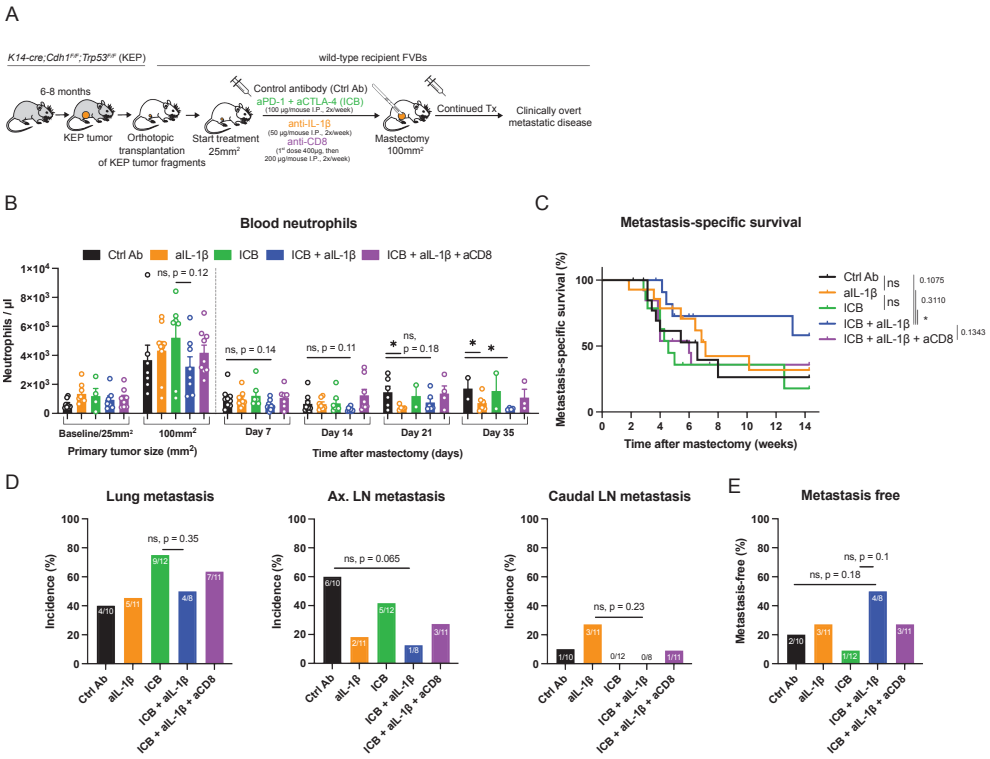


Figure 3. Effect of anti-IL-1β during neoadjuvant ICB in KEP-based metastasis model
(A) Experimental set-up of KEP-based mastectomy model for spontaneous multi-organ metastatic disease and treatment scheme. Treatments were initiated in the neo-adjuvant setting and continued after primary tumor resection until mice developed clinically overt metastatic disease. (B) Absolute neutrophil counts (CD11b^{+} Ly6C^{int} GR1^{high}) in the blood of mice treated as indicated, analyzed by flow cytometry at indicated timepoint, (n=2-9). Mean ± SEM, Unpaired t-test. (C) Kaplan-Meier survival curves of metastasis-bearing mice treated with Ctrl Ab (n=15, 5 censored), anti-IL-1β (n=14, 3 censored), ICB (n=14, 4 censored), ICB + aIL-1β (n=12, 4 censored), or ICB + aIL-1β + aCD8 (n=14, 3 censored). Log-rank (Mantel-Cox) test. (D) Incidence of mice with metastases in the lungs, axillary TDLN (Ax. LN) or caudal lymph node in mice treated as indicated. Mice sacrificed for metastasis-unrelated causes were excluded from the analysis. Fisher's Exact Test. (E) Percentage of mice free of metastasis 100 days after mastectomy. Fisher's Exact Test. ns, not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<000.1.

Exploring new immunotherapeutic strategies for breast cancer

Hundreds of clinical trials are currently testing different combinations of immunotherapeutic strategies in breast cancer patients, sometimes obtaining encouraging results, sometimes not. Clinical implementation seems to be outpacing our mechanistic understanding of which subgroups of patients may

respond and which combination therapies should be rationally explored. As new immunotherapeutic strategies are developed, it will be important to assess the mechanisms of response to these new therapeutics in clinically relevant mouse models to identify possible resistance mechanisms and combination partners that may overcome these hurdles. In **chapter 3 – 5** we identified various ways to improve the established immunotherapy approach of combined anti-PD-1 + anti-CTLA-4 (ICB), either by combining with chemotherapeutic agent cisplatin or engagement of eosinophils, or via simultaneous targeting of tumor-induced immunosuppression. In **chapter 6** we investigated a new immunotherapy approach, which combines an old interest of the immune oncology field with a new one: PD1-IL2v, a newly developed immuno-modulatory agent that aims to deliver an IL-2 variant, engineered to preferentially bind the IL2Rβγ subunit expressed on $CD8^+$ T cells and NK cells over IL2Rα-expressing T_{regs} and endothelial cells, specifically to $PD\text{-}1^+$ tumor-reactive $CD8^+$ T cells using a PD-1-targeted antibody. Murinized (mu)PD1-IL2v showed promising results in pancreatic cancer models expressing strong tumor antigens[3-5], but had not yet been tested in lowly immunogenic and sparsely T cell infiltrated cancer models such as breast cancer. Thus, we set out to put muPD1-IL2v to the test in our highly immunosuppressed KEP mammary tumor model.

The most striking observation coming out of this study was that muPD1-IL2v monotherapy elicited a strong increase in tumor infiltration of $CD8^+$ T cells with a less-differentiated phenotype, increased ability to proliferate and produce IFNγ and TNFα, similar to what had been reported in the pancreatic cancer models[3-5], yet these changes were to no avail. muPD1-IL2v monotherapy did not improve tumor control or survival of KEP mice. Although KEP tumors are certainly lowly immunogenic compared to other tumor models, the complete absence of recognizable tumor antigens is an unlikely explanation for the resistance to muPD1-IL2v monotherapy, given that we can induce anti-tumor/anti-metastasis control in absence of chemotherapy when ICB is combined with T_{reg}-targeting (**chapter 3**) or neutrophil-targeting (**chapter 4**) or when eosinophils are engaged by recombinant IL-33 therapy (**chapter 2**). Thus, a more likely explanation seems that the remaining immunosuppressive cell populations, including T_{regs}, neutrophils, and macrophages, play a dominate role the TME limiting anti-tumor immunity.

Like our experience with aPD-1/CTLA-4 therapy in **chapter 3**, combining muPD1-IL2v with cisplatin induced synergistic anti-tumor control. Cisplatin may be required to release additional tumor antigens or to sensitize tumors to T cell killing. Another explanation may lie in our observation that combined cisplatin and muPD1-IL2v induces a broader intratumoral and systemic engagement of anti-tumor immunity beyond the effect that also muPD1-IL2v monotherapy induced in $CD8^+$ T cells. Indeed, CIS + muPD1-IL2v provoked the additional systemic expansion of NK cells, $CD4^+$ T cells and eosinophils and caused reprogramming of tumor-infiltrating macrophages to an anti-tumorigenic phenotype, possibly contributing to anti-tumor immunity via increase antigen presentation or direct tumoricidal activity. Our preliminary studies identified that $CD8^+$ T cells are the main drivers of the anti-tumor response. Intriguingly, NK cell depletion during CIS + muPD1-IL2v did not affect its therapeutic benefit, but instead prevented the therapy induced weight loss, suggesting NK cells contribute to treatment induced toxicity rather than tumor

control. The functional involvement of increased $CD4^+$ T cells, eosinophils and TAMs remains to be evaluated in future studies. Next steps may also involve evaluating whether reducing the frequency of cisplatin dosing may prevent treatment-related toxicity while retaining therapeutic benefit of combined CIS + muPD1-IL2v. Overall, our data highlight that combining PD1-IL2v with cisplatin is a powerful approach to improve response against lowly immunogenic, immunosuppressed KEP mammary tumors.

Concluding remarks

Our research demonstrates that combining unbiased profiling of patient samples with mechanistic studies in clinically relevant mouse models is a powerful approach to uncover new mechanisms of immunotherapy response and resistance. Our data emphasize that to further understand and improve immunotherapy for breast cancer patients, a shift in perspective of immunotherapy from its traditional emphasis on T cells to a more encompassing view of tumor immunity as an interconnected system, will be needed. By taking such a holistic approach, looking at the crosstalk between innate and adaptive immunity both in the tumor micro- and macro-environment, we identified several key players and their interconnectivities in anti-tumor immunity and tumor-induced immunosuppression that may be therapeutically exploited to improve immunotherapy responses for breast cancer patients.

References

1. Garner, H., and de Visser, K.E. (2020). Immune crosstalk in cancer progression and metastatic spread: a complex conversation. Nat Rev Immunol *20*, 483-497.
2. Chen, D.S., and Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. Immunity *39*, 1-10.
3. Codarri Deak, L., Nicolini, V., Hashimoto, M., Karagianni, M., Schwalie, P.C., Lauener, L., Varypataki, E.M., Richard, M., Bommer, E., Sam, J., et al. (2022). PD-1-cis IL-2R agonism yields better effectors from stem-like CD8+ T cells. Nature *610*, 161-172.
4. Piper, M., Hoen, M., Darragh, L.B., Knitz, M.W., Nguyen, D., Gadwa, J., Durini, G., Karakoc, I., Grier, A., Neupert, B., et al. (2023). Simultaneous targeting of PD-1 and IL-2Rβγ with radiation therapy inhibits pancreatic cancer growth and metastasis. Cancer Cell *41*, 950-969.e6.
5. Tichet, M., Wullschleger, S., Chryplewicz, A., Fournier, N., Marcone, R., Kauzlaric, A., Homicsko, K., Deak, L.C., Umaña, P., Klein, C., and Hanahan, D. (2023). Bispecific PD1-IL2v and anti-PD-L1 break tumor immunity resistance by enhancing stem-like tumor-reactive CD8+ T cells and reprogramming macrophages. Immunity *56*, 162-179.e166.
6. Rosenberg, H.F., Dyer, K.D., and Foster, P.S. (2013). Eosinophils: changing perspectives in health and disease. Nat Rev Immunol *13*, 9-22.
7. Grisaru-Tal, S., Itan, M., Klion, A.D., and Munitz, A. (2020). A new dawn for eosinophils in the tumour microenvironment. Nat Rev Cancer *20*, 594-607.
8. Grisaru-Tal, S., Itan, M., Grass, D.G., Torres-Roca, J., Eschrich, S.A., Gordon, Y., Dolitzky, A., Hazut, I., Avlas, S., Jacobsen, E.A., et al. (2020). Primary tumors from mucosal barrier organs drive unique eosinophil infiltration patterns and clinical associations. Oncoimmunology *10*, 1859732.
9. Kratochvill, F., Neale, G., Haverkamp, J.M., Van de Velde, L.A., Smith, A.M., Kawauchi, D., McEvoy, J., Roussel, M.F., Dyer, M.A., Qualls, J.E., and Murray, P.J. (2015). TNF Counterbalances the Emergence of M2 Tumor Macrophages. Cell Rep *12*, 1902-1914.
10. Zaynagetdinov, R., Sherrill, T.P., Gleaves, L.A., McLoed, A.G., Saxon, J.A., Habermann, A.C., Connelly, L., Dulek, D., Peebles, R.S., Jr., Fingleton, B., et al. (2015). Interleukin-5 facilitates lung metastasis by modulating the immune microenvironment. Cancer Res *75*, 1624-1634.
11. Arnold, I.C., Artola-Boran, M., Gurtner, A., Bertram, K., Bauer, M., Frangez, Z., Becher, B., Kopf, M., Yousefi, S., Simon, H.U., et al. (2020). The GM-CSF-IRF5 signaling axis in eosinophils promotes antitumor immunity through activation of type 1 T cell responses. J Exp Med *217*, e20190706.
12. Carretero, R., Sektioglu, I.M., Garbi, N., Salgado, O.C., Beckhove, P., and Hammerling, G.J. (2015). Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells. Nat Immunol *16*, 609-617.
13. Jia, S., Li, W., Liu, P., and Xu, L.X. (2019). A role of eosinophils in mediating the anti-tumour effect of cryo-thermal treatment. Sci Rep *9*, 13214.
14. Hollande, C., Boussier, J., Ziai, J., Nozawa, T., Bondet, V., Phung, W., Lu, B., Duffy, D., Paradis, V., Mallet, V., et al. (2019). Inhibition of the dipeptidyl peptidase DPP4 (CD26) reveals IL-33-dependent eosinophil-mediated control of tumor growth. Nat Immunol *20*, 257-264.
15. Reichman, H., Itan, M., Rozenberg, P., Yarmolovski, T., Brazowski, E., Varol, C., Gluck, N., Shapira, S., Arber, N., Qimron, U., et al. (2019). Activated Eosinophils Exert Antitumorigenic Activities in Colorectal Cancer. Cancer Immunol Res *7*, 388-400.
16. Andreone, S., Spadaro, F., Buccione, C., Mancini, J., Tinari, A., Sestili, P., Gambardella, A.R., Lucarini, V., Ziccheddu, G., Parolini, I., et al. (2019). IL-33 Promotes CD11b/CD18-Mediated Adhesion of Eosinophils to Cancer Cells and Synapse-Polarized Degranulation Leading to Tumor Cell Killing. Cancers (Basel) *11*, 1664.
17. Lucarini, V., Ziccheddu, G., Macchia, I., La Sorsa, V., Peschiaroli, F., Buccione, C., Sistigu, A., Sanchez, M., Andreone, S., D'Urso, M.T., et al. (2017). IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils. Oncoimmunology *6*, e1317420.
18. Grisaru-Tal, S., Dulberg, S., Beck, L., Zhang, C., Itan, M., Hediyeh-Zadeh, S., Caldwell, J., Rozenberg, P., Dolitzky, A., Avlas, S., et al. (2021). Metastasis-Entrained Eosinophils Enhance Lymphocyte-Mediated Antitumor Immunity. Cancer Res *81*, 5555-5571.
19. Simon, S.C.S., Hu, X., Panten, J., Grees, M., Renders, S., Thomas, D., Weber, R., Schulze, T.J., Utikal, J., and Umansky, V. (2020). Eosinophil accumulation predicts response to melanoma treatment with immune checkpoint inhibitors. Oncoimmunology *9*, 1727116.
20. Delyon, J., Mateus, C., Lefeuvre, D., Lanoy, E., Zitvogel, L., Chaput, N., Roy, S., Eggermont, A.M., Routier, E., and Robert, C. (2013). Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. Ann Oncol *24*, 1697-1703.
21. Gebhardt, C., Sevko, A., Jiang, H., Lichtenberger, R., Reith, M., Tarnanidis, K., Holland-Letz, T., Umansky, L., Beckhove, P., Sucker, A., et al. (2015). Myeloid Cells and Related Chronic Inflammatory Factors as Novel Predictive Markers in Melanoma Treatment with Ipilimumab. Clin Cancer Res *21*, 5453-5459.
22. Alves, A., Dias, M., Campainha, S., and Barroso, A. (2021). Peripheral blood eosinophilia may be a prognostic biomarker in non-small cell lung cancer patients treated with immunotherapy. J Thorac Dis *13*, 2716-2727.
23. Okauchi, S., Shiozawa, T., Miyazaki, K., Nishino, K., Sasatani, Y., Ohara, G., Kagohashi, K., Sato,

S., Kodama, T., Satoh, H., and Hizawa, N. (2021). Association between peripheral eosinophils and clinical outcomes in patients with non-small cell lung cancer treated with immune checkpoint inhibitors. Pol Arch Intern Med *131*, 152-160.
24. Verhaart, S.L., Abu-Ghanem, Y., Mulder, S.F., Oosting, S., Van Der Veldt, A., Osanto, S., Aarts, M.J.B., Houtsma, D., Peters, F.P.J., Groenewegen, G., et al. (2020). Real-world Data of Nivolumab for Patients With Advanced Renal Cell Carcinoma in the Netherlands: An Analysis of Toxicity, Efficacy, and Predictive Markers. Clin Genitourin Cancer *19*, 274.e1-274.e16.
25. Long, H., Liao, W., Wang, L., and Lu, Q. (2016). A Player and Coordinator: The Versatile Roles of Eosinophils in the Immune System. Transfus Med Hemother *43*, 96-108.
26. Ling, M.F., and Luster, A.D. (2016). Allergen-Specific CD4(+) T Cells in Human Asthma. Ann Am Thorac Soc *13 Suppl 1*, S25-30.
27. Pelaia, C., Paoletti, G., Puggioni, F., Racca, F., Pelaia, G., Canonica, G.W., and Heffler, E. (2019). Interleukin-5 in the Pathophysiology of Severe Asthma. Front Physiol *10*, 1514.
28. Akuthota, P., Wang, H., and Weller, P.F. (2010). Eosinophils as antigen-presenting cells in allergic upper airway disease. Curr Opin Allergy Clin Immunol *10*, 14-19.
29. Jacobsen, E.A., Ochkur, S.I., Pero, R.S., Taranova, A.G., Protheroe, C.A., Colbert, D.C., Lee, N.A., and Lee, J.J. (2008). Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. J Exp Med *205*, 699-710.
30. Jacobsen, E.A., Zellner, K.R., Colbert, D., Lee, N.A., and Lee, J.J. (2011). Eosinophils regulate dendritic cells and Th2 pulmonary immune responses following allergen provocation. J Immunol *187*, 6059-6068.
31. Kanda, A., Fleury, S., Kobayashi, Y., Tomoda, K., Julia, V., and Dombrowicz, D. (2015). Th2-activated eosinophils release Th1 cytokines that modulate allergic inflammation. Allergol Int *64 Suppl*, S71-73.
32. Zheng, X., Zhang, N., Qian, L., Wang, X., Fan, P., Kuai, J., Lin, S., Liu, C., Jiang, W., Qin, S., et al. (2020). CTLA4 blockade promotes vessel normalization in breast tumors via the accumulation of eosinophils. Int J Cancer *146*, 1730-1740.
33. Litchfield, K., Reading, J.L., Puttick, C., Thakkar, K., Abbosh, C., Bentham, R., Watkins, T.B.K., Rosenthal, R., Biswas, D., Rowan, A., et al. (2021). Meta-analysis of tumor- and T cell-intrinsic mechanisms of sensitization to checkpoint inhibition. Cell *184*, 596-614.e514.
34. Liang, Y.K., Deng, Z.K., Chen, M.T., Qiu, S.Q., Xiao, Y.S., Qi, Y.Z., Xie, Q., Wang, Z.H., Jia, S.C., Zeng, D., and Lin, H.Y. (2021). CXCL9 Is a Potential Biomarker of Immune Infiltration Associated With Favorable Prognosis in ER-Negative Breast Cancer. Front Oncol *11*, 710286.
35. Gardner, A., de Mingo Pulido, Á., Hänggi, K., Bazargan, S., Onimus, A., Kasprzak, A., Conejo-Garcia, J.R., Rejniak, K.A., and Ruffell, B. (2022). TIM-3 blockade enhances IL-12-dependent antitumor immunity by promoting CD8(+) T cell and XCR1(+) dendritic cell spatial co-localization. J Immunother Cancer *10*, e003571
36. Spranger, S., Dai, D., Horton, B., and Gajewski, T.F. (2017). Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. Cancer Cell *31*, 711-723.e714.
37. House, I.G., Savas, P., Lai, J., Chen, A.X.Y., Oliver, A.J., Teo, Z.L., Todd, K.L., Henderson, M.A., Giuffrida, L., Petley, E.V., et al. (2020). Macrophage-Derived CXCL9 and CXCL10 Are Required for Antitumor Immune Responses Following Immune Checkpoint Blockade. Clin Cancer Res *26*, 487-504.
38. Wu, D., Molofsky, A.B., Liang, H.E., Ricardo-Gonzalez, R.R., Jouihan, H.A., Bando, J.K., Chawla, A., and Locksley, R.M. (2011). Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science *332*, 243-247.
39. Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183-194.
40. Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol *11*, 889-896.
41. Kanda, A., Yun, Y., Bui, D.V., Nguyen, L.M., Kobayashi, Y., Suzuki, K., Mitani, A., Sawada, S., Hamada, S., Asako, M., and Iwai, H. (2021). The multiple functions and subpopulations of eosinophils in tissues under steady-state and pathological conditions. Allergol Int *70*, 9-18.
42. Mesnil, C., Raulier, S., Paulissen, G., Xiao, X., Birrell, M.A., Pirottin, D., Janss, T., Starkl, P., Ramery, E., Henket, M., et al. (2016). Lung-resident eosinophils represent a distinct regulatory eosinophil subset. J Clin Invest *126*, 3279-3295.
43. Abdala-Valencia, H., Coden, M.E., Chiarella, S.E., Jacobsen, E.A., Bochner, B.S., Lee, J.J., and Berdnikovs, S. (2018). Shaping eosinophil identity in the tissue contexts of development, homeostasis, and disease. J Leukoc Biol *104*, 95-108.
44. Gurtner, A., Borrelli, C., Gonzalez-Perez, I., Bach, K., Acar, I.E., Núñez, N.G., Crepaz, D., Handler, K., Vu, V.P., Lafzi, A., et al. (2023). Active eosinophils regulate host defence and immune responses in colitis. Nature *615*, 151-157.
45. Yousefi, S., Gold, J.A., Andina, N., Lee, J.J., Kelly, A.M., Kozlowski, E., Schmid, I., Straumann, A., Reichenbach, J., Gleich, G.J., and Simon, H.U. (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med *14*, 949-953.
46. Pfirschke, C., Engblom, C., Gungabeesoon, J., Lin, Y., Rickelt, S., Zilionis, R., Messemaker, M., Siwicki, M., Gerhard, G.M., Kohl, A., et al. (2020). Tumor-Promoting Ly-6G(+) SiglecF(high) Cells Are Mature and Long-Lived Neutrophils. Cell Rep *32*, 108164.

47. Chan, B.C.L., Lam, C.W.K., Tam, L.S., and Wong, C.K. (2019). IL33: Roles in Allergic Inflammation and Therapeutic Perspectives. Front Immunol *10*, 364.
48. Shen, J.X., Liu, J., and Zhang, G.J. (2018). Interleukin-33 in Malignancies: Friends or Foes? Front Immunol *9*, 3051.
49. Li, A., Herbst, R.H., Canner, D., Schenkel, J.M., Smith, O.C., Kim, J.Y., Hillman, M., Bhutkar, A., Cuoco, M.S., Rappazzo, C.G., et al. (2019). IL-33 Signaling Alters Regulatory T Cell Diversity in Support of Tumor Development. Cell Rep *29*, 2998-3008.e2998.
50. Pastille, E., Wasmer, M.H., Adamczyk, A., Vu, V.P., Mager, L.F., Phuong, N.N.T., Palmieri, V., Simillion, C., Hansen, W., Kasper, S., et al. (2019). The IL-33/ST2 pathway shapes the regulatory T cell phenotype to promote intestinal cancer. Mucosal Immunol *12*, 990-1003.
51. Kos, K., Aslam, M.A., van de Ven, R., Wellenstein, M.D., Pieters, W., van Weverwijk, A., Duits, D.E.M., van Pul, K., Hau, C.S., Vrijland, K., et al. (2022). Tumor-educated Tregs drive organ-specific metastasis in breast cancer by impairing NK cells in the lymph node niche. Cell Rep *38*, 110447.
52. Hung, L.Y., Tanaka, Y., Herbine, K., Pastore, C., Singh, B., Ferguson, A., Vora, N., Douglas, B., Zullo, K., Behrens, E.M., et al. (2020). Cellular context of IL-33 expression dictates impact on anti-helminth immunity. Sci Immunol *5*, eabc6259.
53. Marx, A.F., Kallert, S.M., Brunner, T.M., Villegas, J.A., Geier, F., Fixemer, J., Abreu-Mota, T., Reuther, P., Bonilla, W.V., Fadejeva, J., et al. (2023). The alarmin interleukin-33 promotes the expansion and preserves the stemness of Tcf-1(+) CD8(+) T cells in chronic viral infection. Immunity *56*, 813-828.e810.
54. Chen, L., Sun, R., Xu, J., Zhai, W., Zhang, D., Yang, M., Yue, C., Chen, Y., Li, S., Turnquist, H., et al. (2020). Tumor-Derived IL33 Promotes Tissue-Resident CD8(+) T Cells and Is Required for Checkpoint Blockade Tumor Immunotherapy. Cancer Immunol Res *8*, 1381-1392.
55. Corria-Osorio, J., Carmona, S.J., Stefanidis, E., Andreatta, M., Ortiz-Miranda, Y., Muller, T., Rota, I.A., Crespo, I., Seijo, B., Castro, W., et al. (2023). Orthogonal cytokine engineering enables novel synthetic effector states escaping canonical exhaustion in tumor-rejecting CD8(+) T cells. Nat Immunol *24*, 869-883. Brog, R.A., Ferry, S.L., Schiebout, C.T., Messier, C.M., Cook, W.J., Abdullah, L., Zou, J., Kumar, P., Sentman, C.L., Frost, H.R., and Huang, Y.H. (2022). Superkine IL-2 and IL-33 Armored CAR T Cells Reshape the Tumor Microenvironment and Reduce Growth of Multiple Solid Tumors. Cancer Immunol Res *10*, 962-977.
57. Forssmann, U., Stoetzer, C., Stephan, M., Kruschinski, C., Skripuletz, T., Schade, J., Schmiedl, A., Pabst, R., Wagner, L., Hoffmann, T., et al. (2008). Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol *181*, 1120-1127.
58. Gigon, L., Fettrelet, T., Yousefi, S., Simon, D., and Simon, H.U. (2023). Eosinophils from A to Z. Allergy.
59. Deacon, C.F. (2020). Dipeptidyl peptidase 4 inhibitors in the treatment of type 2 diabetes mellitus. Nat Rev Endocrinol *16*, 642-653.
60. Oikonomopoulou, K., Brinc, D., Kyriacou, K., and Diamandis, E.P. (2013). Infection and cancer: revaluation of the hygiene hypothesis. Clin Cancer Res *19*, 2834-2841.
61. Darani, H.Y., and Yousefi, M. (2012). Parasites and cancers: parasite antigens as possible targets for cancer immunotherapy. Future Oncol *8*, 1529-1535.
62. Osinaga, E. (2007). Expression of cancer-associated simple mucin-type O-glycosylated antigens in parasites. IUBMB Life *59*, 269-273.
63. Yousefi, M., Akbari, M., Hadipour, M., Dehkordi, A.B., Farahbakhsh, Z., and Darani, H.Y. (2023). Parasites as potential targets for cancer immunotherapy. J Cancer Res Clin Oncol *149*, 8027-8038.
64. Alexander, M.P., Fiering, S.N., Ostroff, G.R., Cramer, R.A., and Mullins, D.W. (2018). Beta-glucan-induced inflammatory monocytes mediate antitumor efficacy in the murine lung. Cancer Immunol Immunother *67*, 1731-1742.
65. Kalafati, L., Kourtzelis, I., Schulte-Schrepping, J., Li, X., Hatzioannou, A., Grinenko, T., Hagag, E., Sinha, A., Has, C., Dietz, S., et al. (2020). Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity. Cell *183*, 771-785.e712.
66. Rittmeyer, D., and Lorentz, A. (2012). Relationship between allergy and cancer: an overview. Int Arch Allergy Immunol *159*, 216-225.
67. Holly, E.A., Eberle, C.A., and Bracci, P.M. (2003). Prior history of allergies and pancreatic cancer in the San Francisco Bay area. Am J Epidemiol *158*, 432-441.
68. Turner, M.C., Chen, Y., Krewski, D., Ghadirian, P., Thun, M.J., and Calle, E.E. (2005). Cancer mortality among US men and women with asthma and hay fever. Am J Epidemiol *162*, 212-221.
69. Schoemaker, M.J., Swerdlow, A.J., Hepworth, S.J., McKinney, P.A., van Tongeren, M., and Muir, K.R. (2006). History of allergies and risk of glioma in adults. Int J Cancer *119*, 2165-2172.
70. Vesterinen, E., Pukkala, E., Timonen, T., and Aromaa, A. (1993). Cancer incidence among 78,000 asthmatic patients. Int J Epidemiol *22*, 976-982.
71. Rivoltini, L., Viggiano, V., Spinazzè, S., Santoro, A., Colombo, M.P., Takatsu, K., and Parmiani, G. (1993). In vitro anti-tumor activity of eosinophils from cancer patients treated with subcutaneous administration of interleukin 2. Role of interleukin 5. Int J Cancer *54*, 8-15.
72. Gatault, S., Delbeke, M., Driss, V., Sarazin, A., Dendooven, A., Kahn, J.E., Lefevre, G., and Capron, M. (2015). IL-18 Is Involved in Eosinophil-Mediated Tumoricidal Activity against a Colon Carcinoma Cell Line by Upregulating LFA-1 and ICAM-1. J Immunol *195*, 2483-2492.

73. Li, H., Xiao, Y., Li, Q., Yao, J., Yuan, X., Zhang, Y., Yin, X., Saito, Y., Fan, H., Li, P., et al. (2022). The allergy mediator histamine confers resistance to immunotherapy in cancer patients via activation of the macrophage histamine receptor H1. Cancer Cell *40*, 36-52.e39.
74. Lin, H., Wei, S., Hurt, E.M., Green, M.D., Zhao, L., Vatan, L., Szeliga, W., Herbst, R., Harms, P.W., Fecher, L.A., et al. (2018). Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade-mediated tumor regression. J Clin Invest *128*, 805-815.
75. Ruffell, B., Chang-Strachan, D., Chan, V., Rosenbusch, A., Ho, C.M., Pryer, N., Daniel, D., Hwang, E.S., Rugo, H.S., and Coussens, L.M. (2014). Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell *26*, 623-637.
76. Tauriello, D.V.F., Palomo-Ponce, S., Stork, D., Berenguer-Llergo, A., Badia-Ramentol, J., Iglesias, M., Sevillano, M., Ibiza, S., Canellas, A., Hernando-Momblona, X., et al. (2018). TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis. Nature *554*, 538-543.
77. Lim, A.R., Rathmell, W.K., and Rathmell, J.C. (2020). The tumor microenvironment as a metabolic barrier to effector T cells and immunotherapy. Elife *9*, e55185.
78. Leone, R.D., and Powell, J.D. (2020). Metabolism of immune cells in cancer. Nat Rev Cancer *20*, 516-531.
79. Barsoum, I.B., Smallwood, C.A., Siemens, D.R., and Graham, C.H. (2014). A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. Cancer Res *74*, 665-674.
80. Weinberg, F., Ramnath, N., and Nagrath, D. (2019). Reactive Oxygen Species in the Tumor Microenvironment: An Overview. Cancers (Basel) *11*, 1191.
81. Fleming, V., Hu, X., Weber, R., Nagibin, V., Groth, C., Altevogt, P., Utikal, J., and Umansky, V. (2018). Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. Front Immunol *9*, 398.
82. Kloosterman, D.J., and Akkari, L. (2023). Macrophages at the interface of the co-evolving cancer ecosystem. Cell *186*, 1627-1651.
83. Noy, R., and Pollard, J.W. (2014). Tumor-associated macrophages: from mechanisms to therapy. Immunity *41*, 49-61.
84. Salvagno, C., Ciampricotti, M., Tuit, S., Hau, C.S., van Weverwijk, A., Coffelt, S.B., Kersten, K., Vrijland, K., Kos, K., Ulas, T., et al. (2019). Therapeutic targeting of macrophages enhances chemotherapy efficacy by unleashing type I interferon response. Nat Cell Biol *21*, 511-521.
85. Kos, K., and de Visser, K.E. (2021). The Multifaceted Role of Regulatory T Cells in Breast Cancer. Annu Rev Cancer Biol *5*, 291-310.
86. Coffelt, S.B., Wellenstein, M.D., and de Visser, K.E. (2016). Neutrophils in cancer: neutral no more. Nat Rev Cancer *16*, 431-446.
87. Coffelt, S.B., Kersten, K., Doornebal, C.W., Weiden, J., Vrijland, K., Hau, C.S., Verstegen, N.J.M., Ciampricotti, M., Hawinkels, L., Jonkers, J., and de Visser, K.E. (2015). IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature *522*, 345-348.
88. Wellenstein, M.D., Coffelt, S.B., Duits, D.E.M., van Miltenburg, M.H., Slagter, M., de Rink, I., Henneman, L., Kas, S.M., Prekovic, S., Hau, C.S., et al. (2019). Loss of p53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis. Nature *572*, 538-542.
89. Kim, R., Hashimoto, A., Markosyan, N., Tyurin, V.A., Tyurina, Y.Y., Kar, G., Fu, S., Sehgal, M., Garcia-Gerique, L., Kossenkov, A., et al. (2022). Ferroptosis of tumour neutrophils causes immune suppression in cancer. Nature *612*, 338-346.
90. Huh, S.J., Liang, S., Sharma, A., Dong, C., and Robertson, G.P. (2010). Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer Res *70*, 6071-6082.
91. Wculek, S.K., and Malanchi, I. (2015). Neutrophils support lung colonization of metastasis-initiating breast cancer cells. Nature *528*, 413-417.
92. Gong, Z., Li, Q., Shi, J., Li, P., Hua, L., Shultz, L.D., and Ren, G. (2023). Immunosuppressive reprogramming of neutrophils by lung mesenchymal cells promotes breast cancer metastasis. Sci Immunol *8*, eadd5204.
93. Xiao, Y., Cong, M., Li, J., He, D., Wu, Q., Tian, P., Wang, Y., Yang, S., Liang, C., Liang, Y., et al. (2021). Cathepsin C promotes breast cancer lung metastasis by modulating neutrophil infiltration and neutrophil extracellular trap formation. Cancer Cell *39*, 423-437.e427.
94. Taylor, N.A., Vick, S.C., Iglesia, M.D., Brickey, W.J., Midkiff, B.R., McKinnon, K.P., Reisdorf, S., Anders, C.K., Carey, L.A., Parker, J.S., et al. (2017). Treg depletion potentiates checkpoint inhibition in claudin-low breast cancer. J Clin Invest *127*, 3472-3483.
95. Solomon, I., Amann, M., Goubier, A., Arce Vargas, F., Zervas, D., Qing, C., Henry, J.Y., Ghorani, E., Akarca, A.U., Marafioti, T., et al. (2020). CD25-T(reg)-depleting antibodies preserving IL-2 signaling on effector T cells enhance effector activation and antitumor immunity. Nat Cancer *1*, 1153-1166.
96. Jin, Y., An, X., Mao, B., Sun, R., Kumari, R., Chen, X., Shan, Y., Zang, M., Xu, L., Muntel, J., et al. (2022). Different syngeneic tumors show distinctive intrinsic tumor-immunity and mechanisms of actions (MOA) of anti-PD-1 treatment. Sci Rep *12*, 3278.
97. Van Damme, H., Dombrecht, B., Kiss, M., Roose, H., Allen, E., Van Overmeire, E., Kancheva, D., Martens, L., Murgaski, A., Bardet, P.M.R., et al. (2021). Therapeutic depletion of CCR8(+) tumor-infiltrating regulatory T cells elicits antitumor immunity and synergizes with anti-PD-1 therapy. J Immunother Cancer *9*, e001749.

98. Bos, P.D., Plitas, G., Rudra, D., Lee, S.Y., and Rudensky, A.Y. (2013). Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. J Exp Med *210*, 2435-2466.
99. Clark, N.M., Martinez, L.M., Murdock, S., deLigio, J.T., Olex, A.L., Effi, C., Dozmorov, M.G., and Bos, P.D. (2020). Regulatory T Cells Support Breast Cancer Progression by Opposing IFN-gamma-Dependent Functional Reprogramming of Myeloid Cells. Cell Rep *33*, 108482.
100. Kos, K., Aslam, M.A., van de Ven, R., Wellenstein, M.D., Pieters, W., van Weverwijk, A., Duits, D.E.M., van Pul, K., Hau, C.S., Vrijland, K., et al. (2022). Tumor-educated T(regs) drive organ-specific metastasis in breast cancer by impairing NK cells in the lymph node niche. Cell Rep *38*, 110447.
101. Adams, S.a.L.S.a.T.D.a.C.D.W.a.D.L.M.a.N.R.a.W.E.P.a.M.H.a.T.K.a. (2017). Phase 2 study of pembrolizumab as first-line therapy for PD-L1–positive metastatic triple-negative breast cancer (mTNBC): Preliminary data from KEYNOTE-086 cohort B. Journal of Clinical Oncology *35*, 1088-1088.
102. Emens, L.A., Cruz, C., Eder, J.P., Braiteh, F., Chung, C., Tolaney, S.M., Kuter, I., Nanda, R., Cassier, P.A., Delord, J.P., et al. (2019). Long-term Clinical Outcomes and Biomarker Analyses of Atezolizumab Therapy for Patients With Metastatic Triple-Negative Breast Cancer: A Phase 1 Study. JAMA Oncol *5*, 74-82.
103. Yu, J., Green, M.D., Li, S., Sun, Y., Journey, S.N., Choi, J.E., Rizvi, S.M., Qin, A., Waninger, J.J., Lang, X., et al. (2021). Liver metastasis restrains immunotherapy efficacy via macrophage-mediated T cell elimination. Nat Med *27*, 152-164.
104. Hiam-Galvez, K.J., Allen, B.M., and Spitzer, M.H. (2021). Systemic immunity in cancer. Nat Rev Cancer *21*, 345-359.
105. Rahim, M.K., Okholm, T.L.H., Jones, K.B., McCarthy, E.E., Liu, C.C., Yee, J.L., Tamaki, S.J., Marquez, D.M., Tenvooren, I., Wai, K., et al. (2023). Dynamic CD8(+) T cell responses to cancer immunotherapy in human regional lymph nodes are disrupted in metastatic lymph nodes. Cell *186*, 1127-1143.e1118.
106. Fransen, M.F., Schoonderwoerd, M., Knopf, P., Camps, M.G., Hawinkels, L.J., Kneilling, M., van Hall, T., and Ossendorp, F. (2018). Tumor-draining lymph nodes are pivotal in PD-1/PD-L1 checkpoint therapy. JCI Insight *3*, e124507.
107. Spitzer, M.H., Carmi, Y., Reticker-Flynn, N.E., Kwek, S.S., Madhireddy, D., Martins, M.M., Gherardini, P.F., Prestwood, T.R., Chabon, J., Bendall, S.C., et al. (2017). Systemic Immunity Is Required for Effective Cancer Immunotherapy. Cell *168*, 487-502.e415.
108. Plitas, G.R., A. Y. (2020). Regulatory T cells in cancer. Annu Rev Cancer Biol *4*, 459-477.
109. Gentles, A.J., Newman, A.M., Liu, C.L., Bratman, S.V., Feng, W., Kim, D., Nair, V.S., Xu, Y., Khuong, A., Hoang, C.D., et al. (2015). The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med *21*, 938-945.
110. Shang, B., Liu, Y., Jiang, S.J., and Liu, Y. (2015). Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep *5*, 15179.
111. Jiang, D., Gao, Z., Cai, Z., Wang, M., and He, J. (2015). Clinicopathological and prognostic significance of FOXP3+ tumor infiltrating lymphocytes in patients with breast cancer: a meta-analysis. BMC Cancer *15*, 727.
112. West, N.R., Kost, S.E., Martin, S.D., Milne, K., Deleeuw, R.J., Nelson, B.H., and Watson, P.H. (2013). Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer *108*, 155-162.
113. Liu, S., Foulkes, W.D., Leung, S., Gao, D., Lau, S., Kos, Z., and Nielsen, T.O. (2014). Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. Breast Cancer Res *16*, 432.
114. Stanton, S.E., Adams, S., and Disis, M.L. (2016). Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. JAMA Oncol *2*, 1354-1360.
115. Bianchini, G., De Angelis, C., Licata, L., and Gianni, L. (2022). Treatment landscape of triple-negative breast cancer - expanded options, evolving needs. Nat Rev Clin Oncol *19*, 91-113.
116. Denkert, C., von Minckwitz, G., Darb-Esfahani, S., Lederer, B., Heppner, B.I., Weber, K.E., Budczies, J., Huober, J., Klauschen, F., Furlanetto, J., et al. (2018). Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol *19*, 40-50.
117. Montler, R., Bell, R.B., Thalhofer, C., Leidner, R., Feng, Z., Fox, B.A., Cheng, A.C., Bui, T.G., Tucker, C., Hoen, H., and Weinberg, A. (2016). OX40, PD-1 and CTLA-4 are selectively expressed on tumor-infiltrating T cells in head and neck cancer. Clin Transl Immunology *5*, e70.
118. Kumagai, S., Koyama, S., Itahashi, K., Tanegashima, T., Lin, Y.T., Togashi, Y., Kamada, T., Irie, T., Okumura, G., Kono, H., et al. (2022). Lactic acid promotes PD-1 expression in regulatory T cells in highly glycolytic tumor microenvironments. Cancer Cell *40*, 201-218.e209.
119. Kumagai, S., Togashi, Y., Kamada, T., Sugiyama, E., Nishinakamura, H., Takeuchi, Y., Vitaly, K., Itahashi, K., Maeda, Y., Matsui, S., et al. (2020). The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. Nat Immunol *21*, 1346-1358.
120. Kavanagh, B., O'Brien, S., Lee, D., Hou, Y., Weinberg, V., Rini, B., Allison, J.P., Small, E.J., and

Fong, L. (2008). CTLA4 blockade expands FoxP3+ regulatory and activated effector CD4+ T cells in a dose-dependent fashion. Blood *112*, 1175-1183.
121. Sharma, A., Subudhi, S.K., Blando, J., Scutti, J., Vence, L., Wargo, J., Allison, J.P., Ribas, A., and Sharma, P. (2019). Anti-CTLA-4 Immunotherapy Does Not Deplete FOXP3(+) Regulatory T Cells (Tregs) in Human Cancers. Clin Cancer Res *25*, 1233-1238.
122. Huang, A.C., Orlowski, R.J., Xu, X., Mick, R., George, S.M., Yan, P.K., Manne, S., Kraya, A.A., Wubbenhorst, B., Dorfman, L., et al. (2019). A single dose of neoadjuvant PD-1 blockade predicts clinical outcomes in resectable melanoma. Nat Med *25*, 454-461.
123. Kamada, T., Togashi, Y., Tay, C., Ha, D., Sasaki, A., Nakamura, Y., Sato, E., Fukuoka, S., Tada, Y., Tanaka, A., et al. (2019). PD-1(+) regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. Proc Natl Acad Sci U S A *116*, 9999-10008.
124. Champiat, S., Dercle, L., Ammari, S., Massard, C., Hollebecque, A., Postel-Vinay, S., Chaput, N., Eggermont, A., Marabelle, A., Soria, J.C., and Ferté, C. (2017). Hyperprogressive Disease Is a New Pattern of Progression in Cancer Patients Treated by Anti-PD-1/PD-L1. Clin Cancer Res *23*, 1920-1928.
125. Marangoni, F., Zhakyp, A., Corsini, M., Geels, S.N., Carrizosa, E., Thelen, M., Mani, V., Prussmann, J.N., Warner, R.D., Ozga, A.J., et al. (2021). Expansion of tumor-associated Treg cells upon disruption of a CTLA-4-dependent feedback loop. Cell *184*, 3998-4015 e3919.
126. Anderson, A.C., Joller, N., and Kuchroo, V.K. (2016). Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity *44*, 989-1004.
127. Zhang, Q., Chikina, M., Szymczak-Workman, A.L., Horne, W., Kolls, J.K., Vignali, K.M., Normolle, D., Bettini, M., Workman, C.J., and Vignali, D.A.A. (2017). LAG3 limits regulatory T cell proliferation and function in autoimmune diabetes. Sci Immunol *2*, eaah4569.
128. Banerjee, H., Nieves-Rosado, H., Kulkarni, A., Murter, B., McGrath, K.V., Chandran, U.R., Chang, A., Szymczak-Workman, A.L., Vujanovic, L., Delgoffe, G.M., et al. (2021). Expression of Tim-3 drives phenotypic and functional changes in Treg cells in secondary lymphoid organs and the tumor microenvironment. Cell Rep *36*, 109699.
129. Zhang, P., Gao, F., Wang, Q., Wang, X., Zhu, F., Ma, C., Sun, W., and Zhang, L. (2007). Agonistic anti-4-1BB antibody promotes the expansion of natural regulatory T cells while maintaining Foxp3 expression. Scand J Immunol *66*, 435-440.
130. Glasner, A., Rose, S.A., Sharma, R., Gudjonson, H., Chu, T., Green, J.A., Rampersaud, S., Valdez, I.K., Andretta, E.S., Dhillon, B.S., et al. (2023). Conserved transcriptional connectivity of regulatory T cells in the tumor microenvironment informs new combination cancer therapy strategies. Nat Immunol *24*, 1020-1035.
131. Salvagno, C., Ciampricotti, M., Tuit, S., Hau, C.S., van Weverwijk, A., Coffelt, S.B., Kersten, K., Vrijland, K., Kos, K., Ulas, T., et al. (2019). Therapeutic targeting of macrophages enhances chemotherapy efficacy by unleashing type I interferon response. Nat Cell Biol *21*, 511-521.
132. Gyori, D., Lim, E.L., Grant, F.M., Spensberger, D., Roychoudhuri, R., Shuttleworth, S.J., Okkenhaug, K., Stephens, L.R., and Hawkins, P.T. (2018). Compensation between CSF1R+ macrophages and Foxp3+ Treg cells drives resistance to tumor immunotherapy. JCI Insight *3*, e120631.
133. Kos, K., Salvagno, C., Wellenstein, M.D., Aslam, M.A., Meijer, D.A., Hau, C.S., Vrijland, K., Kaldenbach, D., Raeven, E.A.M., Schmittnaegel, M., et al. (2022). Tumor-associated macrophages promote intratumoral conversion of conventional CD4(+) T cells into regulatory T cells via PD-1 signalling. Oncoimmunology *11*, 2063225.
134. Binnewies, M., Mujal, A.M., Pollack, J.L., Combes, A.J., Hardison, E.A., Barry, K.C., Tsui, J., Ruhland, M.K., Kersten, K., Abushawish, M.A., et al. (2019). Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4(+) T Cell Immunity. Cell *177*, 556-571 e516.
135. Obradovic, A., Ager, C., Turunen, M., Nirschl, T., Khosravi-Maharlooei, M., Iuga, A., Jackson, C.M., Yegnasubramanian, S., Tomassoni, L., Fernandez, E.C., et al. (2023). Systematic elucidation and pharmacological targeting of tumor-infiltrating regulatory T cell master regulators. Cancer Cell *41*, 933-949.e11.
136. De Simone, M., Arrigoni, A., Rossetti, G., Gruarin, P., Ranzani, V., Politano, C., Bonnal, R.J.P., Provasi, E., Sarnicola, M.L., Panzeri, I., et al. (2016). Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells. Immunity *45*, 1135-1147.
137. Plitas, G., Konopacki, C., Wu, K., Bos, P.D., Morrow, M., Putintseva, E.V., Chudakov, D.M., and Rudensky, A.Y. (2016). Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer. Immunity *45*, 1122-1134.
138. Campbell, J.R., McDonald, B.R., Mesko, P.B., Siemers, N.O., Singh, P.B., Selby, M., Sproul, T.W., Korman, A.J., Vlach, L.M., Houser, J., et al. (2021). Fc-Optimized Anti-CCR8 Antibody Depletes Regulatory T Cells in Human Tumor Models. Cancer Res *81*, 2983-2994.
139. Kidani, Y., Nogami, W., Yasumizu, Y., Kawashima, A., Tanaka, A., Sonoda, Y., Tona, Y., Nashiki, K., Matsumoto, R., Hagiwara, M., et al. (2022). CCR8-targeted specific depletion of clonally expanded Treg cells in tumor tissues evokes potent tumor immunity with long-lasting memory. Proc Natl Acad Sci U S A *119*, e2114282119
140. Boivin, G., Faget, J., Ancey, P.B., Gkasti, A., Mussard, J., Engblom, C., Pfirschke, C., Contat, C., Pascual, J., Vazquez, J., et al. (2020). Durable and controlled depletion of neutrophils in mice. Nat Commun *11*, 2762.
141. Olofsen, P.A., Stip, M.C., Jansen, J.H.M., Chan, C., Nederend, M., Tieland, R.G., Tsioumpekou,

M., and Leusen, J.H.W. (2022). Effective, Long-Term, Neutrophil Depletion Using a Murinized Anti-Ly-6G 1A8 Antibody. Cells *11*, 3406.
142. Crawford, J., Ozer, H., Stoller, R., Johnson, D., Lyman, G., Tabbara, I., Kris, M., Grous, J., Picozzi, V., Rausch, G., and et al. (1991). Reduction by granulocyte colony-stimulating factor of fever and neutropenia induced by chemotherapy in patients with small-cell lung cancer. N Engl J Med *325*, 164-170.
143. Steele, C.W., Karim, S.A., Leach, J.D.G., Bailey, P., Upstill-Goddard, R., Rishi, L., Foth, M., Bryson, S., McDaid, K., Wilson, Z., et al. (2016). CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma. Cancer Cell *29*, 832-845.
144. Highfill, S.L., Cui, Y., Giles, A.J., Smith, J.P., Zhang, H., Morse, E., Kaplan, R.N., and Mackall, C.L. (2014). Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. Sci Transl Med *6*, 237ra267.
145. Sagiv, J.Y., Michaeli, J., Assi, S., Mishalian, I., Kisos, H., Levy, L., Damti, P., Lumbroso, D., Polyansky, L., Sionov, R.V., et al. (2015). Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell Rep *10*, 562-573.
146. Evrard, M., Kwok, I.W.H., Chong, S.Z., Teng, K.W.W., Becht, E., Chen, J., Sieow, J.L., Penny, H.L., Ching, G.C., Devi, S., et al. (2018). Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. Immunity *48*, 364-379. e368.
147. Mitchem, J.B., Brennan, D.J., Knolhoff, B.L., Belt, B.A., Zhu, Y., Sanford, D.E., Belaygorod, L., Carpenter, D., Collins, L., Piwnica-Worms, D., et al. (2013). Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. Cancer Res *73*, 1128-1141.
148. Qian, B.Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L.R., Kaiser, E.A., Snyder, L.A., and Pollard, J.W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature *475*, 222-225.
149. Bonapace, L., Coissieux, M.M., Wyckoff, J., Mertz, K.D., Varga, Z., Junt, T., and Bentires-Alj, M. (2014). Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. Nature *515*, 130-133.
150. Nywening, T.M., Belt, B.A., Cullinan, D.R., Panni, R.Z., Han, B.J., Sanford, D.E., Jacobs, R.C., Ye, J., Patel, A.A., Gillanders, W.E., et al. (2018). Targeting both tumour-associated CXCR2(+) neutrophils and CCR2(+) macrophages disrupts myeloid recruitment and improves chemotherapeutic responses in pancreatic ductal adenocarcinoma. Gut *67*, 1112-1123.
151. Kumar, V., Donthireddy, L., Marvel, D., Condamine, T., Wang, F., Lavilla-Alonso, S., Hashimoto, A., Vonteddu, P., Behera, R., Goins, M.A., et al. (2017). Cancer-Associated Fibroblasts Neutralize the Anti-tumor Effect of CSF1 Receptor Blockade by Inducing PMN-MDSC Infiltration of Tumors. Cancer Cell *32*, 654-668 e655.
152. Swierczak, A., Cook, A.D., Lenzo, J.C., Restall, C.M., Doherty, J.P., Anderson, R.L., and Hamilton, J.A. (2014). The promotion of breast cancer metastasis caused by inhibition of CSF-1R/CSF-1 signaling is blocked by targeting the G-CSF receptor. Cancer Immunol Res *2*, 765-776.
153. Beffinger, M., Tallon de Lara, P., Tugues, S., Vermeer, M., Montagnolo, Y., Ohs, I., Cecconi, V., Lucchiari, G., Gagliardi, A., Misljencevic, N., et al. (2018). CSF1R-dependent myeloid cells are required for NK-mediated control of metastasis. JCI Insight *3*, e97792
154. Kim, K., Skora, A.D., Li, Z., Liu, Q., Tam, A.J., Blosser, R.L., Diaz, L.A., Jr., Papadopoulos, N., Kinzler, K.W., Vogelstein, B., and Zhou, S. (2014). Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. Proc Natl Acad Sci U S A *111*, 11774-11779.
155. Teijeira, Á., Garasa, S., Gato, M., Alfaro, C., Migueliz, I., Cirella, A., de Andrea, C., Ochoa, M.C., Otano, I., Etxeberria, I., et al. (2020). CXCR1 and CXCR2 Chemokine Receptor Agonists Produced by Tumors Induce Neutrophil Extracellular Traps that Interfere with Immune Cytotoxicity. Immunity *52*, 856-871.e858.
156. Hirschhorn, D., Budhu, S., Kraehenbuehl, L., Gigoux, M., Schröder, D., Chow, A., Ricca, J.M., Gasmi, B., De Henau, O., Mangarin, L.M.B., et al. (2023). T cell immunotherapies engage neutrophils to eliminate tumor antigen escape variants. Cell *186*, 1432-1447.e1417.
157. Gungabeesoon, J., Gort-Freitas, N.A., Kiss, M., Bolli, E., Messemaker, M., Siwicki, M., Hicham, M., Bill, R., Koch, P., Cianciaruso, C., et al. (2023). A neutrophil response linked to tumor control in immunotherapy. Cell *186*, 1448-1464.e1420.
158. Kersten, K., Coffelt, S.B., Hoogstraat, M., Verstegen, N.J.M., Vrijland, K., Ciampricotti, M., Doornebal, C.W., Hau, C.S., Wellenstein, M.D., Salvagno, C., et al. (2017). Mammary tumor-derived CCL2 enhances pro-metastatic systemic inflammation through upregulation of IL1β in tumor-associated macrophages. Oncoimmunology *6*, e1334744.
159. Lee, J.M., Tsuboi, M., Kim, E.S., Mok, T.S., and Garrido, P. (2022). Overcoming immunosuppression and pro-tumor inflammation in lung cancer with combined IL-1β and PD-1 inhibition. Future Oncol *18*, 3085-3100.
160. Nakamura, K., and Smyth, M.J. (2017). Targeting cancer-related inflammation in the era of immunotherapy. Immunol Cell Biol *95*, 325-332.