Dissertation

Regulation of immune cells in the tumor microenvironment: the role of IL-33 and 2-AG

submitted by

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Graz, November 25, 2021

DISCLOSURES

This cumulative dissertation is based on the following papers:

1. **Melanie Kienzl**[1,2], Carina Hasenoehrl[1], Paulina Valadez-Cosmes[1], Kathrin Maitz[1], Arailym Sarsembayeva[1], Eva Sturm[1], Akos Heinemann[1,2], Julia Kargl[1] & Rudolf Schicho[1,2] (2020) IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils, OncoImmunology, 9:1, DOI: 10.1080/2162402X.2020.1776059 (1)
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3. **Melanie Kienzl**[1,2], Carina Hasenoehrl[1], Kathrin Maitz[1], Arailym Sarsembayeva[1], Ulrike Taschler[3], Paulina Valadez-Cosmes[1], Oliver Kindler[1], Dusica Ristic[1], Sofia Raftopoulou[1], Ana Santiso[1], Thomas Baernthaler[1], Luka Brcic[4], Lisa Hahnefeld[5], Robert Gurke[5,6], Dominique Thomas[5], Gerd Geisslinger[5,6], Julia Kargl[1] & Rudolf Schicho[1,2] (2021) Monoacylglycerol lipase deficiency in the tumor microenvironment slows tumor growth in non-small cell lung cancer. OncoImmunology, 10:1, DOI: 10.1080/2162402X.2021.1965319 (3)

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Julia Kargl – planned and co-supervised the projects, contributed to data interpretation and manuscript editing.
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All co-authors have agreed to the inclusion of their published data in the dissertation and permission from respective publishers and the copyright holders for reproduction has been obtained.

I further contributed to the following publications during my PhD studies:

- Valadez-Cosmes, P., Maitz, K., Kindler, O., Raftopoulou, S., **Kienzl, M**., Santiso, A., Mihalic, Z. N., Brcic, L., Lindenmann, J., Fediuk, M., Pichler, M., Schicho, R., Houghton, A. M., Heinemann, A., & Kargl, J. (2021). Identification of Novel Low-Density Neutrophil Markers Through Unbiased High-Dimensional Flow Cytometry Screening in Non-Small Cell Lung Cancer Patients. Front Immunol, 12, 703846. https://doi.org/10.3389/fimmu.2021.703846
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- Knuplez E, **Kienzl M**, Trakaki A, Schicho R, Heinemann A, Sturm EM, Marsche G. The anti-parasitic drug miltefosine suppresses activation of human eosinophils and ameliorates allergic inflammation in mice. Br J Pharmacol. 2021 Mar;178(5):1234-1248. doi: 10.1111/bph.15368.
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- Grill M, Hasenoehrl C, **Kienzl M**, Kargl J, Schicho R. Cellular localization and regulation of receptors and enzymes of the endocannabinoid system in intestinal and systemic inflammation. Histochem Cell Biol. 2019 Jan;151(1):5-20. doi: 10.1007/s00418-018-1719-0.

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TABLE OF CONTENTS

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
2-OG	2-oleoylglycerol
AA	arachidonic acid
ABHD	alpha beta hydrolase domain protein
AEA	anandamide
AOM	azoxymethane
BMDE	bone marrow derived eosinophils
CB	cannabinoid receptor
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
CRC	colorectal cancer
DC	dendritic cell
DSS	dextran sulfate sodium
ECM	extracellular matrix
ECS	endocannabinoid system
FFA	free fatty acid
ICI	immune checkpoint inhibitor therapy
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
LPC	lysophosphatidylcholine
MDSC	myeloid derived suppressor cell
MGL	monoacylglycerol lipase
MMP	matrix metalloproteinase
NK	natural killer cell
NSCLC	non-small-cell lung cancer
OEA	oleoylethanolamide
PC	phosphatidylcholine
PEA	palmitoylethanolamide
PMN	Polymorphonuclear neutrophils
PPARγ	peroxisome proliferator-activated receptor γ
qRT-PCR	quantitative real time polymerase chain reaction
s.c.	subcutaneous
Siglec	sialic acid-binding immunoglobulin-type lectins
SMA	smooth muscle actin
ST2	suppression of tumorgenicity
T regs	regulatory T cells
TAN	tumor-associated neutrophil
TCR	T cell receptor
TGF	transforming growth factor
TME	tumor microenvironment
TNF	tumor necrosis factor
VEA	cis-vaccenic acid ethanolamide
VEGF	vascular endothelial growth factor

ZUSAMMENFASSUNG

Tumorzellen existieren in einem organähnlichen System von komplexen Zellinteraktionen, das als Tumormikroumgebung (TMU) bezeichnet wird. Die TMU besteht aus einer Kombination von Immunzellen, nicht-immunen Stromazellen und einer Fülle von azellulären Komponenten, die gemeinsam zur Tumorentwicklung beitragen. Durch die Beeinflussung der Form der TMU können diese azellulären Komponenten das empfindliche Gleichgewicht, das für die Tumorkontrolle verantwortlich ist, in Richtung Immun-Überwachung oder -Entkommen verschieben. Diese kumulative Dissertation konzentriert sich auf den Einfluss löslicher Mediatoren auf Komponenten der TMU und ihre Auswirkungen auf das Tumorwachstum. Die untersuchten Mediatoren üben nachweislich starke immunmodulatorische Funktionen aus. Das Zytokin IL-33 und Komponenten des Endocannabinoidsystems (ECS), mit dem Schwerpunkt auf Monoacylglycerol-Lipase (MGL) und dessen Substrat 2-Arachidonoylglycerol (2-AG), einem bekannten Endocannabinoid, werden in den drei enthaltenen Publikationen behandelt:

- In der ersten Veröffentlichung untersuchten wir die immunmodulatorische Wirkung von IL-33 in einem heterotopen und chemisch-induzierten Colitis-assoziierten Darmkrebsmodell (CRC). In diesen Modellen reduzierte die Behandlung von Mäusen mit IL-33 das Tumorwachstum. Die Verringerung der Tumorgröße war von der Anwesenheit eosinophiler Granulozyten in der TMU abhängig. Darüber hinaus konnten wir zeigen, dass die IL-33-Aktivierung direkt zu einer erhöhten Überlebensrate, Degranulation und Migration von eosinophilen Granulozyten führte.

- Komponenten des ECS sind für ihre Fähigkeit bekannt Immunzellfunktionen zu vermitteln, aber auch das Verhalten von Tumorzellen direkt zu beeinflussen. In diesem umfassenden Übersichtsartikel wird die Literatur über das ECS im Zusammenhang mit der TMU untersucht und es werden mögliche Auswirkungen auf die Tumorprogression beleuchtet. (Endo)cannabinoide können potente Mediatoren sein, die den Zustand der TMU beeinflussen.

- Die Rolle der MGL-Expression in der Tumorpathogenese wurde umfassend untersucht, ihr Einfluss auf die TMU ist jedoch nach wie vor nicht klar. Wir haben ein heterotopes Modell für nicht-kleinzelligen Lungenkrebs (NSCLC) in einer MGL-defizienten Maus verwendet, um die Auswirkungen von MGL und dessen Substrat 2-AG auf den Zustand der TMU zu untersuchen. Interessanterweise verringerte die Abwesenheit von MGL in der TMU das Wachstum des NSCLC und ging mit einer erhöhten Konzentration von 2-AG und einer Zunahme der eosinophilen Granulozyten und $CD8^+$ T-Effektorzellen einher. Wir fanden heraus, dass 2-AG

die Rekrutierung und Aktivierung von eosinophilen Granulozyten und $CD8^+$ T-Zellen *in vitro* vermittelt und somit zu einer tumorwachstums-verringernden TMU beiträgt.

Zusammenfassend lässt sich sagen, dass die untersuchten Mediatoren vielversprechende Ziele für die Induktion eines antitumorigenen Milieus sind und eine Option als Adjuvans für Immuntherapien darstellen könnten.

ABSTRACT

Tumors exist in an organ-like system with a variety of non-tumor cells with complex cell interactions, termed the tumor microenvironment (TME). The TME comprises a combination of immune cells, non-immune stromal cells, and a plethora of acellular components, which can contribute to tumor development. By influencing the shape of the TME, those acellular components may shift the delicate balance of tumor control towards immune surveillance or escape. This cumulative dissertation focuses on the influence of soluble mediators on components of the TME and their effects on tumor progression. In particular, the investigated mediators have been shown to exert potent immunomodulatory functions. The alarmin IL-33 and components of the endocannabinoid system (ECS), with a focus on the 2-arachidonoylglycerol (2-AG)/monoacylglycerol lipase (MGL)-axis, are addressed in the three included publications:

- In the first publication we explored the immunomodulatory effect of IL-33 in a heterotopic and chemically induced colitis-associated colorectal cancer (CRC) model. IL-33 treatment of mice reduced the tumor growth of CRC in both models. Importantly, tumor size reduction was dependent on the presence of eosinophils in the TME. Furthermore, we showed that IL-33 activation directly resulted in increased survival, degranulation, and migration of eosinophils towards CRC.

- Components of the ECS are known for their ability to mediate immune cell functions but also directly affect tumor cell behavior. Herein, a comprehensive review article explores the literature about the ECS in the context of the TME and examines possible effects on tumor progression. (Endo)cannabinoids may be potent mediators influencing the state of the TME.

- The role of MGL expression in tumor pathogenesis, has been comprehensively studied, however, its influence in the TME remains elusive. We used a heterotopic non-small cell lung cancer (NSCLC) model in a MGL deficient mouse to investigate the effects of MGL and its substrate 2-AG on the state of the TME. Interestingly, MGL deficiency in the TME reduced NSCLC growth and was accompanied by increased levels of 2-AG and enhanced eosinophil and $CD8^+$ T effector cells. We found that 2-AG mediates recruitment and activation of eosinophils and $CD8^+$ T cells *in vitro,* thus contributing to an anti-tumorigenic TME.

In summary, the investigated mediators are promising targets for the induction of an anti-tumorigenic environment and may be an option as adjuvants during immunotherapy.

1 INTRODUCTION

1.1 Cancer - one of the leading causes of death worldwide

Together with cardiovascular disease, cancer is leading the way as a cause for premature deaths (4). In 2020, lung and colon cancer accounted for the highest numbers of new cancer cases worldwide, with only breast cancer being diagnosed more often. With 18 %, however, lung cancer is the leading cause of cancer deaths, followed by colon cancer with 9.4 % of all cancer deaths (5).

Lung cancer is sub-classified into two major histological types: small cell lung carcinoma, comprising about 15 % of all lung cancers, and non-small cell lung cancer (NSCLC), accounting for the other 85 % (6). NSCLC is further categorized into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (6). The major CRC type is the adenocarcinoma (more than 90 %), which arises from epithelial cells in the colon and rectum. It is followed by rare types like squamous cell carcinoma and spindle cell carcinoma, among others (7).

As to lung cancer, a range of risk factors has been described, especially highlighting the influence of tobacco smoking, because reduced consumption is followed by a decline in incidence and vice versa (8). Moreover, e.g. inheritance of certain single-nucleotide-polymorphisms, diet and alcohol, chronic inflammation, and air pollution were accounted as risk factors for lung cancer development (8). The majority of CRC cases develop sporadically, mainly through risk factors, such as poor diet, low physical activity, alcohol and obesity, followed by hereditary factors, such as family history, causing hereditary cancer syndromes (9,10).

Those risk factors contribute to the development of cancerous cells. Transformation into malignant cells is caused by replication errors, environmental exposure and/or oncoviruses, which result in genetic and epigenetic alterations, and in chromosomal aberrations in normal cells. Together, these events may contribute to the genesis of cancer (11).

1.2 The tumor microenvironment - immune surveillance and escape

Aberrant cells are constantly recognized and destroyed by cells of the immune system in a process called immune surveillance (12). However, the development of tumor cell variants, being able to hide from the immune system - due to events like genetic instability or immune selection and suppression - might ultimately facilitate the escape of tumor cells and lead to cancer progression (13).

Nonetheless, next to cancer cells, not only immune cells, but also non-immune stromal cells and acellular components comprise the niche, called tumor microenvironment (TME), which altogether contribute to cancer development (14). As such, the TME is a heterogenous assortment that comprises immune cells, blood vessels and cells of the lymphatic system, fibroblasts, cancer stem cells, pericytes and adipocytes, signaling molecules, mediators and extracellular matrix (ECM) components that are released by TME cells (15).

The TME provides a niche for the tumor to arrange a milieu for angiogenesis to supply the proliferating cancer cells with nutrients and oxygen, to overcome hypoxia and nutrient deprivation, but also to remove metabolic waste (16). Infiltration of immune cells, on the one hand, supports the fight against the disease, but on the other hand, it might also support tumor progression. According to the tumor entity, certain TME components might vary in their presence, however blood vessels, leukocytes, stromal cells, and extracellular matrix components are major tumor characteristics (16). The TME is classified as a tumor hallmark, however the immune landscape can vary drastically between cancer types and contributes to patient survival (17). Interestingly, different driver mutations in malignant cells correlated with differing infiltrating leukocyte numbers and populations (17,18).

1.2.1 Cells of the tumor microenvironment in tumor progression

$CD8^+$ T cells are known as the main tumor killing cells (19). After antigen encounter by antigen presenting cells, naïve $CD8^+$ T cells differentiate into T effector and memory cells (20). Aberrant antigen presentation on major histocompatibility complex (MHC) I, resulting from overexpressed or mutated protein translation in cancerous cells is harnessed by antigen-specific cytotoxic $CD8^+$ T cells to identify tumor cells (21). Together with NK cells, $CD4^+$ T helper cells of the type 1 phenotype, eosinophils, and antigen presenting cells, like dendritic cells (DCs), contribute to an anti-tumorigenic TME, resulting in suppression of tumor growth (22–26). $CD4^+$ T helper 1 (Th1) cells mediate anti-tumor effects via assisting $CD8^+$ T cells (22), similarly to DCs, by presenting antigens to $CD8^+$ T cells, hence, activating anti-tumor immunity (23). Importantly, tumor cells may alter the expression of MHC-I molecules on their surface to “hide” from $CD8^+$ T cells (27). NK cells, however, are known to kill cells with aberrant or absent expression of MHC-I (28). Furthermore, eosinophils have been shown to either kill tumor cells directly by releasing their cytotoxic granule proteins (29,30), or they support the recruitment and activation of e.g. $CD8^+$ T and NK cells (31–33).

Moreover, the TME is additionally populated with tumor supporting immune cells, like neutrophils, tumor associated macrophages, myeloid derived suppressor cells, type 2 $CD4^+$ helper cells, and regulatory T cells, which support tumor progression, invasion, and metastasis

(16). Tumor promoting tasks include the suppression of anti-tumorigenic immune cells, the promotion of blood vessel formation for enhanced nutrient supply, and facilitating invasion by remodeling of the ECM (34). Non-immune stromal cells including cancer-associated fibroblasts, adipocytes, endothelial cells, pericytes and adipocytes complete the list of cells in the TME that can support tumor development (34).

Taken together, different components of the TME, on the one hand, drive immune evasion, metastasis, and provide the tumor with oxygen and nutrients to promote tumor growth, but on the other hand, these components support the host in the fight against the malignant disease, trying to keep tumor progression at bay (16). At this point, it has to be stressed, that the above-mentioned functions of intratumoral leukocytes only describe a general picture. The specific role of these cells, however, is influenced by a milieu of soluble factors; hence, functions of immune cells in the TME may largely differ depending on the composition of the soluble microenvironment (34,35).

1.2.2 Soluble mediators in the tumor microenvironment

As mentioned above, the TME not only comprises cellular components, but also ECM proteins and soluble mediators, that contribute to tumor development. Communication within the TME and subsequent actions are mediated either directly through cell-cell contact or by soluble mediators through autocrine or paracrine signaling. Soluble mediators include e.g. cytokines, growth factors, chemokines, and metabolites of distinct origins that contribute to a pro- or anti-tumorigenic phenotype of the TME (Figure 1)(36).

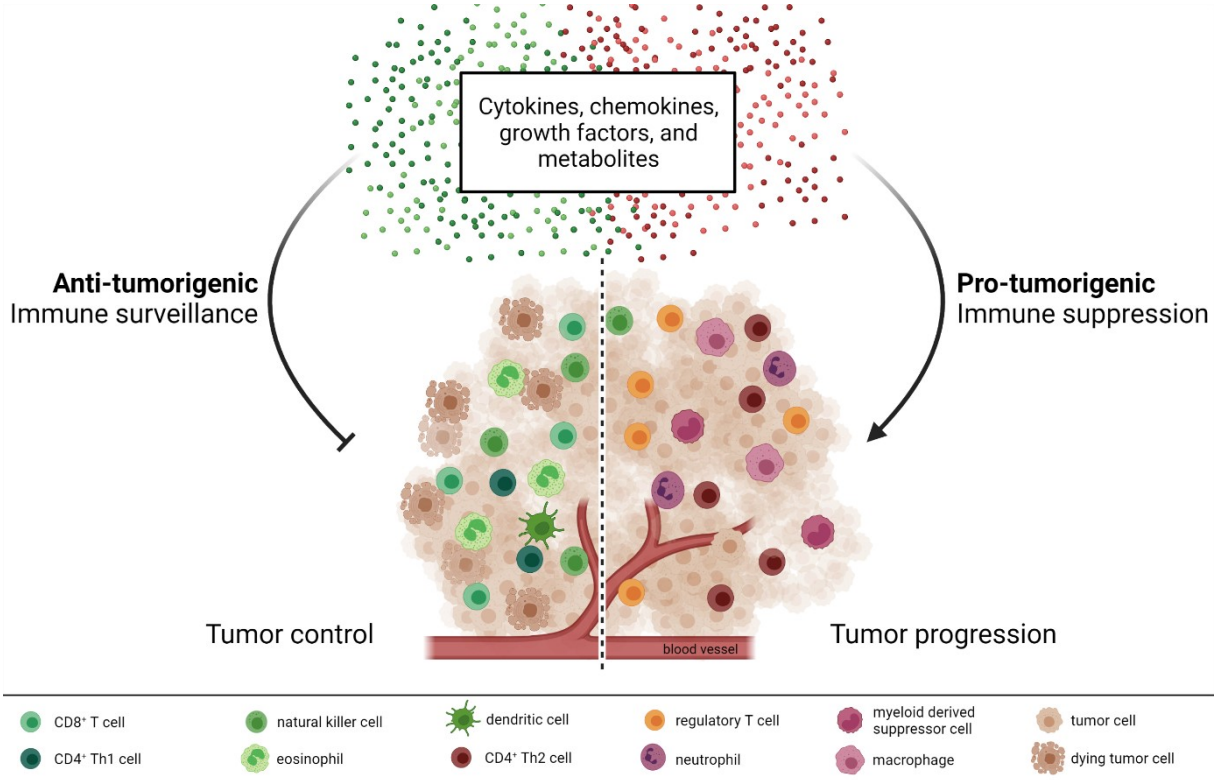

Figure 1 Soluble mediators influence cells of the tumor microenvironment (TME). Cytokines, chemokines, growth factors, and metabolites (including endocannabinoids) are soluble mediators derived from various cells of the TME (and also tumor cells). They shape the cell landscape in the TME of cancer, contributing to immune surveillance or suppression, thus contributing to tumor development. Created with Biorender.com.

1.2.2.1 Cytokines, chemokines, growth factors

Cytokines, chemokines and growth factors influence the tumor development by playing an important role in the communication of different cell types within the TME (36). Cytokines can support tumor growth and metastasis, but they can also activate anti-tumorigenic immunity and inhibit tumor development by signaling through their receptors on target cells (37). As such, a tumor promoting role for cytokines like IL-4, IL-6, IL-8, IL-10 and TGF-β is supported by the literature (38,39). In contrast, IL-2 and related interleukins, like IL-15 and IL-21, stimulate the immune system to fight off the cancer (40–42). Nevertheless, the page is not always black or white for the role of cytokines in tumor development (43,44). This is especially true for IL-33, an alarmin, whose role in the TME is strongly debated (45). IL-33 will be the focus of chapter 1.3.1 in this thesis.

Certain immune cell types that exemplify the influence of the cytokine milieu in the TME, and consequently tumor progression, are the following: (1) macrophages, (2) neutrophils, and (3) eosinophils.

1) *Tumor-associated macrophages (TAMs)*

TAMs are found in the stroma of tumors from different origins (46). Their role in tumor progression, however, is contradictory. On the one hand, elevated numbers of TAMs have been reported to be connected with poor prognosis in human cancers (47,48). On the other hand, improved survival in, e.g., colon (47,49) and gastric cancer (50) was associated with increased TAMs.

A better understanding of their influence on tumor progression was achieved through the identification of their functional state which is dependent on the soluble mediators in the environment they reside in (46). Thus, macrophage subtypes are divided in M1 (classically-activated) or M2 (alternatively-activated) cells, depending on effector functions and the factors needed for polarization (51,52). Polarization into the M1 phenotype is induced by lipopolysaccharide and type 1 cytokine signaling (like by TNF-α and IFN-γ) while polarization towards M2 is mediated by type 2 cytokines (like IL-4, IL13) (53,54). M1 polarized macrophages have been reported to exert their anti-tumorigenic functions, for example, directly by killing cancer cells (55,56), or indirectly via shaping the TME (57) and contributing to vessel normalization (57,58). In contrast to that, M2 macrophages are linked to suppression of immunity (59) as well as enhanced expression of tissue remodeling, repair (60,61), and pro-angiogenic molecules (62,63).

2) *Neutrophils*

Until recently, the role of neutrophils in cancer was understated (64), although, with up to 20 %, neutrophils depict the most abundant immune cell type in NSCLC (65). Neutrophils represent also a substantial percentage of the infiltrating leukocytes in other solid cancers (66). Neutrophil infiltration into NSCLC leads to reduction of $CD4^+$ and $CD8^+$ T cells, and a low $CD8^+$/PMN ratio predicts immune checkpoint inhibitor therapy (ICI) deficiency (67). Nevertheless, tumor-associated neutrophils have been reported to be a favorable prognostic factor in gastric and colorectal cancer (68,69).

The studies mentioned above highlight a contradictory role of neutrophils in cancer. Consequently, Fridlender et al. proposed a certain plasticity between a pro- and anti-tumorigenic state (70). A pro-tumorigenic type of neutrophils (N2) was observed in the presence of TGF-β, whereas blockade of TGF-β or the presence of type I IFNs induced anti-tumorigenic neutrophils (N1) (70–72). Thus, depleting tumor-associated neutrophils (TANs) or targeting TAN-released pro-tumorigenic molecules was suggested as an adjuvant to immunotherapy by Zhang and Houghton (73).

3) *Eosinophils*

Eosinophils mainly localize in the stroma (74) and necrotic areas of tumors (75). They have been reported to exert pro- or anti-tumorigenic effects (76). Therefore, Varricchi et al. suggested an influence of different tumor entities on eosinophil function, which might be due to differences of the surrounding milieu (77). In line with this suggestion, Nazaroff and colleagues recently reported two distinct immune phenotypes of eosinophils with different effector functions (78). The polarization of type 1 eosinophils (E1) is induced by TNF-α and IFN-γ, whereas the generation of type 2 eosinophils (E2) is stimulated by IL-4, GMCSF and IL-33 (78). E1 eosinophils were indicated as favorable for lung allograft acceptance (79) and E2 eosinophils were shown to contribute to allergic responses (80). Interestingly, both immune phenotypes were described in experimental cancer models (76). However, the role of a pro- or anti-tumorigenic function is less well characterized than in the above-mentioned cell types, highlighting the importance of research in this field.

Other soluble mediators that influence the TME are chemokines that lead to the recruitment of distinct leukocyte populations, thus, contributing to the distinct immune cell composition of the TME (67,81). On the one hand, chemokines recruit leukocytes to the site of the tumor, resulting in tumor control or progression depending on the cell type attracted, but on the other hand, they can also bind to receptors on tumor and stromal cells inducing survival, proliferation and angiogenesis (82).

Growth factors, such as vascular endothelial growth factor (VEGF) and EGF contribute to angiogenesis and cell proliferation, differentiation, and migration, respectively (36), thus, contributing to tumor progression.

For more detailed information about chemokines and growth factors in the TME, the reader is forwarded to a review by Nagarsheth and colleagues (82) and by Witsch and colleagues (83), respectively.

1.2.2.2 Metabolites

The Warburg effect largely explains the changed metabolism of cancer cells, which is shifted towards glycolysis and enhanced lactate fermentation irrespective of oxygen availability (84). The acidification induced by increased lactate in the TME stimulates polarization of macrophages towards the pro-tumorigenic M2 phenotype and increased VEGF and TGF-β production by those cells (85). Increased glycolysis furthermore reduces glucose accessibility in the tumor tissue, which, in turn, suppresses immune cell functions (86).

Moreover, tumors not only have an increased need for glucose, but also require fatty acids as building blocks for new membranes during proliferation (87). As such, free fatty acid (FFA)-

producing enzymes, such as monoacylglycerol lipase (MGL) or phospholipase A_2, were reported to be increased in malignant cells (88,89). However, those enzymes not only provide a pool of FFAs for tumor growth, but they are also important for the generation of eicosanoids including prostaglandins and leukotrienes as these enzymes generate their precursor molecule arachidonic acid (AA) (89,90). Prostaglandins and leukotrienes either influence the growth of cancer cells directly, or mediate their, mostly, tumor promoting effects through stromal cells of the TME (91). As such, prostaglandin E_2 signaling induces a downregulation of type 1 and upregulation of type 2 cytokines in leukocytes, resulting in a shift towards an immunosuppressive TME (92). Furthermore, direct inhibition of $CD8^+$ T cells (93) and M2 macrophage polarization (94) was reported for PGE_2. Additionally to immunosuppression, eicosanoids are also involved in angiogenesis by stimulating the release of VEGF from immune cells (91).

MGL, however, not only plays a role in providing building blocks for proliferating cancer cells and AA for the production of pro-inflammatory eicosanoids, but it is also the major enzyme for the degradation of the endocannabinoid 2-AG, subsequently limiting its availability (95). This is of importance, as 2-AG is known as an immunomodulator and, thus, might play a role in shaping the TME (2). The role of the MGL/2-AG axis in the TME will be discussed in detail in chapter 1.3.2.

Additionally, metabolites including L-arginine, adenosine, and kynurenine are known to influence immune cells in the TME (34). As such, depletion of L-arginine, through arginase expressed by cells like TAMs and N2 neutrophils (96), leads to reduced T cell proliferation (97). The degradation of adenosine monophosphate to adenosine by tumor-derived extracellular vesicles might lead to reduced cytotoxic T and NK cell function (98). Indoleamine 2,3-dioxygenase expression by immune cells, like eosinophils, leads to the catalyzation of tryptophan to kynurenine, which is known to suppress T cells and blunt immunity (76,99).

In summary, the above-mentioned soluble mediators influence the milieu of the TME by various mechanisms, such as recruiting pro- or anti-tumorigenic leukocytes, suppressing or activating immune cells, thus shifting the balance of immunosurveillance in the one or other direction.

1.3 Pro-/Anti-tumorigenic soluble mediators that affect the shape of the tumor microenvironment

In this chapter I will focus on two soluble mediators in the TME that are very different in their origin, but may, at least in parts, result in similar effects on the composition of the TME and, hence, on tumor development.

1.3.1 IL-33 - alarmin(g) cytokine of the tumor microenvironment

IL-33 is a nuclear cytokine that is part of the IL-1 family (100). Two isoforms of IL-33 have been described, namely full-length and mature IL-33 (101). For example, neutrophil or mast cell-derived proteases cleave the full-length IL-33 into a mature IL-33, thus increasing its bioactivity approximately 30-fold (102,103). It is released by fibroblasts, epithelial and endothelial cells in response to cell stress, tissue injury or necrosis, acting as an alarmin in the surrounding tissue (100,104). Engagement of IL-33 with its receptor ST2 induces signaling pathways leading to activation of target cells (101). ST2 is expressed by innate immune cells, including eosinophils (105), mast cells (106), macrophages (107), basophils (108), DCs (109), and innate lymphoid cells (ILC2) (110), but also by cells of the adaptive immune system, like $CD4^+$ (111) and $CD8^+$ T cells (112), NKT and NK cells (113). Furthermore, ST2 expression was detected in various cancer cells including CRC and ovarian cancer (114,115). The availability of IL-33 in the TME is regulated by the expression of a soluble ST2 receptor (sST2) from, e.g., cancer cells, epithelial cells and leukocytes, thereby competing for IL-33 with the membrane-localized ST2, decreasing its effects (116,117). This emphasizes IL-33 as an important modulator of the TME.

1.3.1.1 IL-33 - implications in tumorigenesis

Elevated IL-33 was detected in various cancer patients, including hepatocellular carcinoma (118), breast (119) and lung cancer (120), correlating with poor prognosis. The IL-33 decoy receptor, sST2, was linked to good prognosis in CRC due to inhibition of tumor progression by blocking IL-33 signaling (121). Interestingly, a pro-tumorigenic role of IL-33 in tumor growth was confirmed in experimental models: genetic deficiency of IL-33 (122) or its receptor ST2 (123,124) blocked tumor cell proliferation, it activated apoptosis and inhibited blood vessel formation, resulting in reduced tumor burden in a spontaneous CRC model ($Apc^{Min/+}$). Treatment of lung cancer cells with IL-33 led to a dose dependent increase in aggressiveness that was associated with matrix metalloproteinase (MMP) 2 and 9 expression (125), and with increased tumor growth in a xenograft model (126).

In contrast to that, IL-33 signaling was also described to have tumor suppressive effects in various cancers (127). As such, ST2 expression was found to be decreased in a highly metastatic lung cancer cell line (128). Furthermore, IL-33 expression correlated negatively with increased stages of lung cancer (128), and it was a factor of good prognosis in osteosarcoma patients (129). Immunohistochemical staining unveiled that IL-33$^+$ tumor cells and cancer-associated fibroblasts indicated good prognosis in cholangiocarcinoma. In the same study, increased migration of a cholangiocarcinoma cell line after IL-33 knockdown was reported (130). Interestingly, a direct inhibition of tumor cell growth by IL-33 was observed in CRC (131,132) and pancreatic cancer cells *in vitro* (133).

1.3.1.2 IL-33 in tumor immune surveillance and escape

As mentioned above, a pleiotropic role of IL-33 in cancer development was observed (127). Hence, response to IL-33 was suggested to be dependent on the tumor entity, the affected leukocyte populations present in the TME, and the interaction with other factors of the TME (134). Various immune cells express ST2 and thus, are capable of responding to IL-33, thereby affecting the TME (101).

CD8$^+$ T cells

IL-33 induces ST2 expression on CD8$^+$ T cells and furthermore enhances IFN-γ production by TCR-stimulated CD8$^+$ T cells in synergy with IL-12, highlighting its role in promoting a Th1 response (135). By expression of IL-33 in B16 melanoma and 4T1 breast cancer cell lines, Gao and colleagues could show reduced primary tumor and metastasis growth due to increased activation, proliferation and infiltration of CD8$^+$ and NK cells (136). Moreover, endogenous IL-33 signaling induced IFN-γ production by CD8$^+$ and CD4$^+$ T cells, thereby reducing tumor growth in the colon by overcoming induced T reg populations (137). In line with these findings, ST2 inhibition reduced CD8$^+$ T cell infiltration and supported tumor progression (132).

CD4$^+$ Tcells

ST2 expression is low on naïve CD4$^+$ T cells, however, TCR activation and cytokine stimuli induces its expression in vitro (138). Furthermore, ST2 is enhanced in a feedback loop by IL-33 (139). Despite the fact that IL-33 signaling is known to induce pro-tumorigenic Th2 polarization in CD4$^+$ T cells (45), Th1-mediated anti-tumor immune responses were also reported. Thus, when used as an adjuvant with an HPV16 E6/7-encoded DNA vaccine, IL-33 enhanced the secretion of IFN-γ and TNF-α by CD4$^+$ T cells (140). In a similar fashion, IFN-γ$^+$ CD4$^+$ T cells were increased in hepatocellular (141) and colon carcinoma (142) through IL-33 signaling, thereby contributing to reduced tumor growth.

T regs

In contrast to their $CD4^+$ counterparts, proliferation of $ST2^+$ T regs was increased by IL-33 of epithelial cells resulting in a Th2-prone environment and increased tumor growth in $Apc^{Min/+}$ mice (124). Comparably, in an subcutaneous (s.c.) CRC model, treatment with IL-33 enhanced, while blocking of IL-33 attenuated the expansion of T regs (143). T reg recruitment was stimulated by IL-33-induced CCL2 production through TGF-β in esophageal squamous cell carcinoma (144). Additionally, T reg specific deficiency of ST2 increased $CD8^+$ T cell infiltration followed by reduced tumor growth, highlighting a role of the IL-33/ST2 axis in T reg-induced immunosuppression in mouse lung cancer (145). In a metastatic melanoma model, IL-33 treatment reduced the cytotoxicity and increased inhibitory molecule expression by $CD8^+$ T cells, and was linked to an increased suppressive environment including T reg accumulation (146). In line with these findings, IFN-γ expression by T cells was reduced after IL-33-mediated T reg expansion in collagen-induced arthritis (147). Collectively, these and other studies suggest a role of IL-33 in T reg proliferation, recruitment, and activation of their immunosuppressive properties, thereby exerting pro-tumorigenic effects (45).

NK cells

IL-33 signaling is involved in NK cell proliferation and protection against viral infections (148). In metastasis models of melanoma and lung cancer, IL-33 expression led to increased NK cell recruitment, resulting in reduced metastasis development (149). Furthermore, a role in IL-33-mediated activation of NK cells with increased IFN-γ and CD107a/IFN-γ expression was reported in breast cancer (136) and melanoma models (33), respectively. However, a pro-tumorigenic role of the IL-33/ST2 axis was also reported. IL-33 treatment of mice challenged with a breast cancer cell line resulted in decreased activity of NK cells, thus supporting tumor progression (150).

Eosinophils

Until recently, eosinophils were mainly reduced to end stage effector cells mediating cytotoxic effects in the defense against helminths, and representing the main infiltrate in an asthmatic lung (151,152). Nevertheless changing perspectives in research enabled the acknowledgement of eosinophils as multi-faceted cells playing an important role in homeostasis, host defense, repair and remodeling mechanisms (153,154). Harboring granules, filled with molecules like eosinophil peroxidase, eosinophil cationic protein, eosinophil derived neurotoxin, granzyme A and B, cytokines, chemokines, lipid mediators and growth factors, eosinophils are regarded as important immune regulators (155,156). As mentioned in a previous chapter, their effector functions are dependent on the surrounding milieu. IL-33, along with IL-4 and GMCSF, was reported to induce an E2 subtype of eosinophils

(78). IL-33/ST2 signaling, thus, induces secretion of type 2 cytokines, but it also increases survival, expression of markers for activation (CD69) and degranulation (CD63), and of adhesion molecules (CD11b and ICAM-1) in eosinophils (30,33,78,105,157,158). In line with these findings, several studies investigated the role of eosinophils in IL-33-induced tumor response. Lucarini et al. observed reduced tumor growth after administration of IL-33, which could be reversed by eosinophil depletion (33). IL-33-induced anti-tumor effects were either mediated through recruitment of cytotoxic T and NK cells to the TME (33) or by direct tumor cell killing (159). Eosinophil-mediated killing was investigated *in vitro* (29). Interestingly, IL-33 stimulation induced the tumoricidal effects of eosinophils (33) and revealed the necessity of adhesion to tumor cells through the formation of an CD11b/CD18 dependent immunological synapse (30) for the killing of tumor cells.

Tumor-associated tissue eosinophilia has been reported to be a factor of good prognosis in CRC patients (160–163). Reichman and colleagues confirmed these clinical findings in an experimental model of CRC by showing that eosinophil-deficient mice developed enhanced tumor burden. They also associated IFN-γ dependent activation of eosinophils to their tumoricidal properties (25). As described above, IL-33 activation was also reported to induce anti-tumorigenic properties of eosinophils in melanoma (30,33). In the current thesis I, therefore, investigated the so far unknown role of IL-33-induced anti-cancer function of eosinophils in CRC.

DCs

DCs depict a rare myeloid cell population in the TME (164). Additionally, they only express low levels of ST2, however IL-33 is still capable of inducing the expression of co-stimulatory molecules, MHC-I and II and cytokine secretion (45), enhancing maturation, cross-priming of cytotoxic T cells, thus contributing to anti-tumor immunity (165).

Basophils

Next to their role in allergic inflammation, basophils have been acknowledged as immunoregulators (reviewed in (166,167)). By expressing ST2 they respond to IL-33 by degranulation and cytokine production (168). The role of basophils in cancer is still elusive (169), however IL-33 stimulation increased expression of granzyme B and CD63, additionally, they showed increased tumoricidal activity against melanoma cells *in vitro* (170).

Macrophages

IL-33 was reported to induce M2 polarization of macrophages in a NSCLC xenograft model *in vivo* and *in vitro* (171), and recruit M2 macrophages in models of CRC (121,124,172) and breast cancer (150), thus inducing tumor progression.

MDSC

Myeloid-derived suppressor cells (MDSCs) can be detected mainly during ongoing inflammation, in both, infection or cancer (173,174). Treatment with IL-33 increased peripheral and also infiltrating MDSCs in a mouse model of breast cancer (150). In contrast to that, MDSC frequencies were reduced in the TME after IL-33 injection in melanoma-bearing mice (33). These studies emphasize that differences in IL-33-mediated MDSC responses occur according to the tumor entity (45).

Neutrophils

The role of IL-33 on neutrophil function in the TME is not well studied (45). A recent study showed that IL-33 expression in metastasis-associated fibroblasts induced the infiltration of leukocytes, including neutrophils, to the metastatic lung in mouse breast cancer (175). Consequences of neutrophil accumulation on metastatic progression were not investigated (175). Nevertheless, in a virus-induced hepatitis model, neutrophil recruitment was increased by IL-33, followed by T cell suppression and protection against liver injury, suggesting immunosuppression through IL-33 signaling (176).

Mast cells

Mast cells are part of the innate immune system and are involved in the pathology of different diseases, including cancer (177). Human mast cell lines showed increased expression of vascular endothelial growth factor (VEGF) which was mediated through substance P in response to IL-33 treatment (178), suggesting an involvement of IL-33-activated mast cells in angiogenesis and tumor development (179). Despite these studies, the role of mast cells in cancer is still elusive (180).

Type 2 innate lymphoid cells (ILC2s)

ILC2s are known to respond to IL-33 with proliferation, recruitment and induction of Th2 responses (181–183). Pro-tumorigenic functions of ILC2s may include upregulation of Th2 cells, MDSCs, and M2 macrophages (184). Moreover, IL-33-mediated expansion of ILC2s led to NK cell suppression (185). In contrast to that, in a model of pancreatic ductal adenocarcinoma, endogenous IL-33 induced ILC2s to recruit DCs, which subsequently attracted and activated $CD8^+$ T cells, thus promoting a tumoricidal response (186).

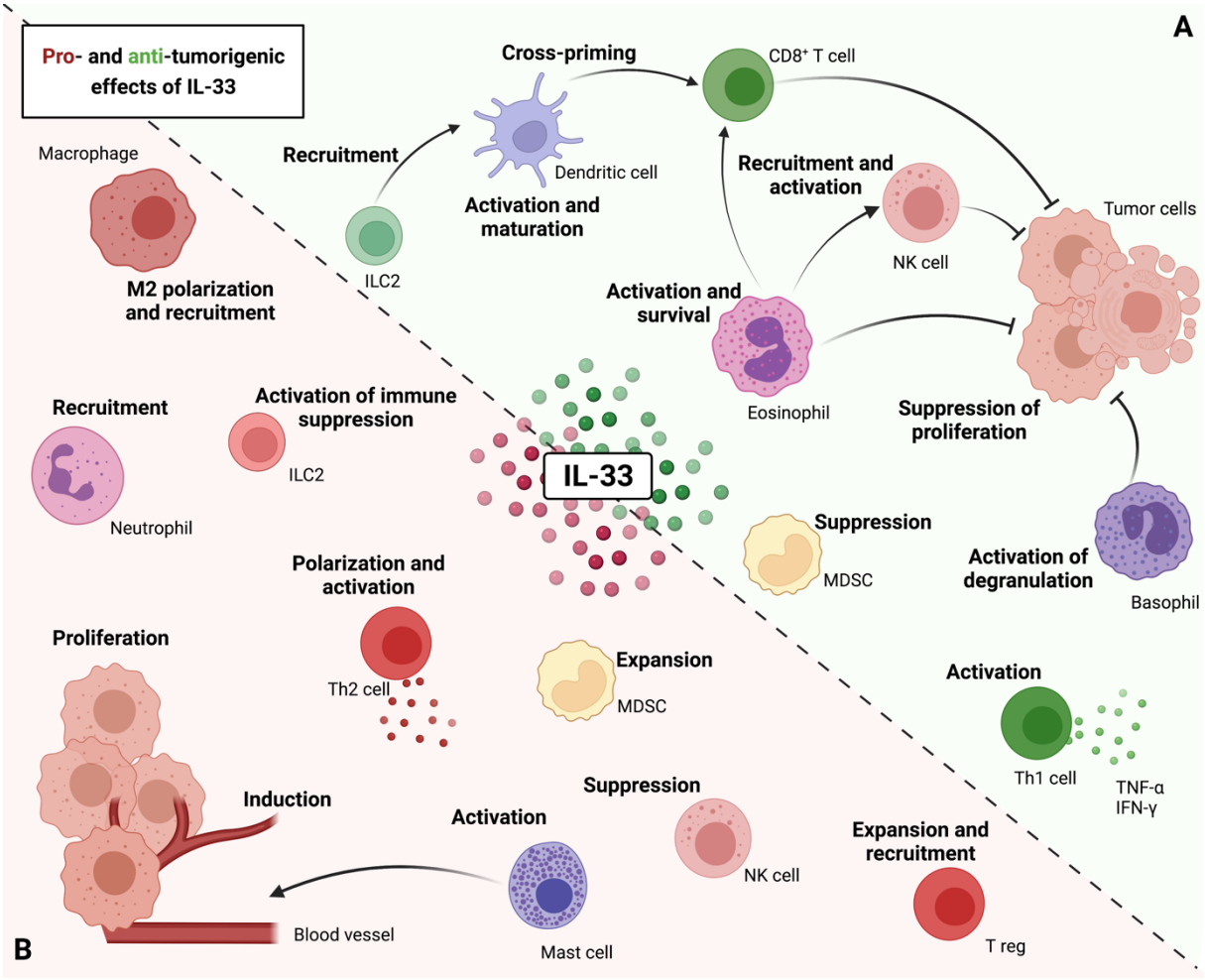

Figure 2 Functions of IL-33 in the tumor microenvironment (TME). In cancer, the alarmin IL-33 is released by cancer cells, endothelial, epithelial cells, or fibroblasts and activates infiltrating immune cell populations, thus inducing a pro- or anti-tumorigenic TME. ILC2-type 2 innate lymphoid cell, MDSC-myeloid derived suppressor cell, NK-natural killer cell. Created with Biorender.com

Taken together, the alarmin IL-33 is a potent tool to shift the phenotype of the TME. However, due to its pleiotropic function (Figure 2) the effect on the infiltrating immune cell populations and the tumor cells is not yet completely understood and might not only depend on its concentration, but also on other soluble factors in the TME (45,127).

1.3.2 2-AG and the immune-endocannabinoid system in the tumor microenvironment

2-AG is not merely a lipid, but it also has been described as an endocannabinoid and thus, it is part of the endocannabinoid system (ECS). The ECS consists of endocannabinoids such as 2-AG and anandamide (AEA), the receptors through which the endocannabinoids signal, their transporters and their synthesizing (diacylglycerol lipase and N-acylphosphatidyl-ethanolamine phospholipase D) and degrading enzymes (monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH)) (187,188). 2-AG is synthesized by consecutive

degradation of phospholipids into diacylglycerol, followed by hydrolysis with diacylglycerol lipase (189). 2-AG is a full agonist of the two cannabinoid receptors 1 (CB_1) and 2 (CB_2) (190), and it was also described to bind to the orphan endocannabinoid receptor GPR55 (191). Furthermore, 2-AG activates receptors of the nuclear hormone receptor PPAR family (192), highlighting its role in the "expanded" ECS or "Endocannabinoidome" (193). CB_1 is predominantly expressed by cells of the central and enteric nervous system (194). In comparison, it is less expressed by cells of the immune system (195). Some types of cancer cells have been described to express CB_1 (196,197). On the other hand, CB_2 is mainly expressed by immune cells (198,199), on the other hand it was additionally reported in cancer cells (200) and fibroblasts (201). Uptake of 2-AG is regulated by diffusion, transmembrane binding proteins or transporters (202), subsequently leading to its hydrolysis into arachidonic acid (AA) and glycerol by MGL, which is the major enzyme known to hydrolyze 2-AG (95). MGL is responsible for 85 % of 2-AG hydrolysis in the brain (203) and, consequently, inhibition or deficiency of MGL leads to 2-AG accumulation (204,205). MGL expression was reported by tumor cells of different entities (88,206) and by cells of the TME, including cancer-associated fibroblasts (3), monocytes (207), macrophages (208), and eosinophils (209). Additionally, 2-AG might also undergo phosphorylation (210), and can be metabolized by cyclooxygenase-2, thus, it contributes to the generation of prostaglandin precursor molecules (211).

The expression of cannabinoid receptors and the major endocannabinoid degrading enzymes MGL and FAAH at the tumor site are summarized in Figure 3.

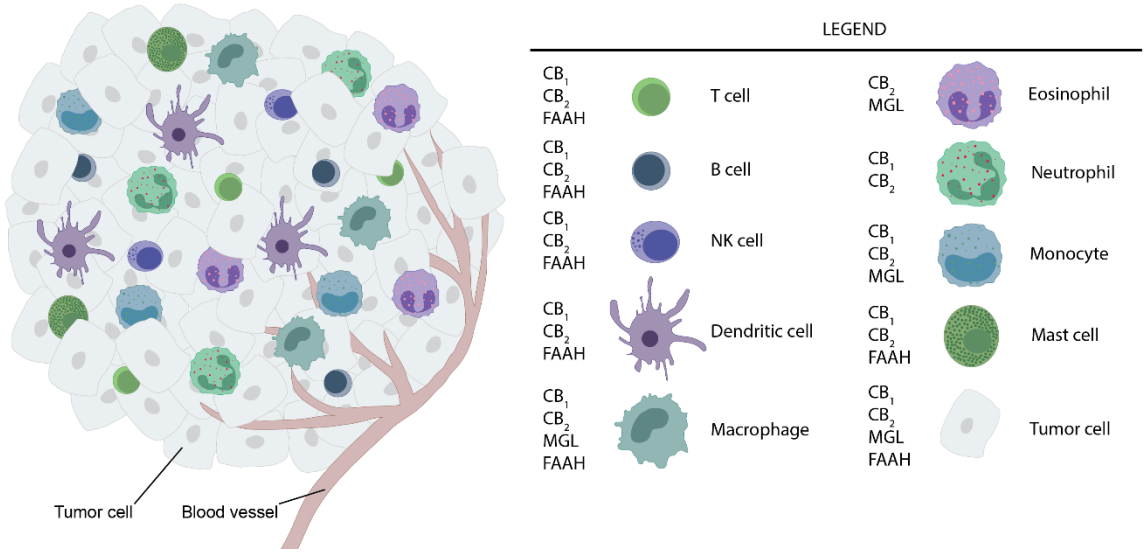

Figure 3 Expression of components of the endocannabinoid system (ECS) by immune cells of the tumor microenvironment (TME). This image summarizes the expression of cannabinoid receptors 1 (CB_1) and 2 (CB_2), as well as the expression of monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) by cellular components of the TME. Image adapted from Kienzl et al. (2) under Creative Commons Attributions License.

Cells of the immune system not only express cannabinoid receptors and synthesizing/degrading enzymes for endocannabinoids, but also endocannabinoids themselves, serving as an important source of endocannabinoid release (2). As such, 2-AG is expressed by lymphocytes (212,213), monocytes (213), macrophages (214,215), DCs (216), neutrophils (213) and eosinophils (213).

Clearly, with components of the ECS being expressed in the TME, it is likely that tumor cells and TME cells (and subsequently tumor progression) are affected by endocannabinoids, in particular by 2-AG.

1.3.2.1 2-AG/MGL-axis affects tumor progression

In the lipolytic pathway, MGL is responsible for hydrolysis of monoacylglycerol into free fatty acids and glycerol (217). The enzyme may provide building blocks for membrane synthesis and signaling molecules for proliferating cancer cells, which have been described to shift their energy metabolism rather towards glycolysis and lipid synthesis (87). As such, MGL expression is linked to reduced survival in lung cancer (218) and hepatocellular carcinoma patients (219). The cancer promoting effects of MGL have further been supported by experimental studies. For example, Nomura et al. observed remodeling of fatty acids in cancer cells with increased activity of MGL resulting in tumorigenesis (88). Furthermore, colon cancer cells react to the use of the MGL inhibitor JZL184 or genetic knockdown of MGL with increased apoptotic cell death and reduced cell division (220,221). Nomura et al. used RNA-interference and pharmacological inhibition of MGL resulting in reduced migration, invasion and survival of human PC3 prostate cancer cells (222). The same was found in human CRC cell lines (221). Moreover, the role of MGL in a gene expression pattern that is related to epithelial-to-mesenchymal transition and stem-like properties of cancer cell was highlighted (222). Interestingly, treatment with the MGL inhibitor JZL184 reduced tumor growth and development of metastasis in a osteosarcoma model (223). In contrast, decreased MGL expression was detected in clinical samples of CRC (224) and squamous cell carcinoma (225). In one of these studies, MGL overexpression reduced the colony formation of a human CRC cell line (224). Furthermore, spontaneous tumor development was observed in aged MGL deficient mice (226). In another study, survival of pancreatic cancer patients did not correlate with MGL expression (227). These, sometimes, contradicting data suggest that the role of MGL in cancer cells is dependent on the type of cancer and other still unknown factors.

One of these factors could be 2-AG which is controlled by MGL activity. Despite its role in tumor cell metabolism, MGL might also affect tumor growth by regulating 2-AG levels in the TME. As mentioned above, inhibition or deficiency of MGL leads to accumulation of 2-AG

(204,205). 2-AG concentrations were increased in the plasma of melanoma (228) and CRC patients (229). Interestingly, 2-AG treatment reduced proliferation of a pancreatic cancer (230) and CRC cell line (231) *in vitro*.

1.3.2.2 2-AG as an immunomodulator

As mentioned in the previous chapter, immune cells express CB_1 and CB_2, making them responsive to 2-AG (232). Thus, 2-AG was shown to decrease IL-2 expression in a T cell line (233). Furthermore, 2-AG was reported to be important in the CB_2-dependent recruitment of B cells (234), NK cells (235), DCs (236), macrophages (237), and eosinophils (238–240). In contrast to that, the role of 2-AG in neutrophil migration is controversial. On the one hand, researchers reported that 2-AG does not serve as a chemoattractant for neutrophils (209,241), but it could suppresses *N*-formyl-l-methionyl-l-leucyl-l-phenylalanine-induced migration (241). On the other hand, Balenga and colleagues described a role for 2-AG in neutrophil migration and polarization that was enhanced upon GPR55 activation (242). It was also shown that 2-AG injection served as an adjuvant to induce a Th1 response to a soluble protein (236). Reduced TNF-α and reactive oxygen species (ROS) intermediate production was detected in mouse macrophages upon 2-AG treatment (243), however, phagocytosis was enhanced (244). Furthermore, actin polymerization in a human macrophage cell line (245), and ROS production of neutrophils was stimulated by 2-AG in a CB_2 dependent manner (242). In contrast, not all 2-AG induced actions are mediated via CB_2. Activation of neutrophils to release myeloperoxidase and mobilize calcium was induced by 2-AG hydrolysis and subsequent leukotriene B_4 synthesis and signaling (209). Additionally, researchers reported an immunomodulatory role of 2-AG on mast cells. As such, histamine release was blunted (246) and LPS-induced TNF-α secretion (247) was reduced upon 2-AG treatment.

In summary, the aforementioned studies support the role of 2-AG as an immunoregulatory molecule, consequently suggesting its part in shaping the TME (2,232). So far only a couple of studies describe the role of 2-AG or MGL in the context of the TME. First, exogenous 2-AG was described to induce the infiltration of MDSCs into an orthotopic pancreatic cancer model and stimulating DC maturation in the spleen (230). Secondly, MGL deficiency in macrophages led to increased tumor growth induced by CB_2 signaling in a CRC model (208). Thus, further research to identify the role of the 2-AG/MGL axis in the TME of various types of cancer is necessary, especially in the context of MGL inhibitors that are investigated in clinical trials as cancer therapies (248).

1.4 Tumor microenvironment in immunotherapies of cancer

In the last three decades, immunotherapies revolutionized the treatment of cancer patients and enhanced prognosis and quality of life (249). The term 'immunotherapy' summarizes different treatment strategies to fight cancer by harnessing the immune system (250). These strategies comprise (i) immunomodulators, (ii) specific and unspecific adoptive cell transfer, (iii) cancer vaccines, and (iv) immune checkpoint inhibitor therapy (ICI) (250–252).

In detail, immunomodulators are artificially produced cytokines, such as IL-2, which aim to induce the antigen presentation and the adaptive immune response (253). Adoptive cell transfer-based therapies are subdivided into two groups, into non-specific adoptive cell therapy including DCs, NKs and tumor-infiltrating lymphocytes, and specific adoptive cell therapy using ex vivo expanded and genetically modified patient-derived chimeric antigen receptor T cells (252), for instance. Cancer vaccines aim to induce an immune response towards cancer specific antigens which in turn can kill tumor cells and furthermore might even develop an immune memory (254).

Lastly, ICI is targeting the off switch of the immune system, which is hijacked by tumor cells to suppress anti-tumorigenic lymphocytes (255–257). ICI targeting the programmed cell death protein 1 (PD-1) and the cytotoxic T lymphocyte-associated antigen (CTLA-4) have been described as most promising for patient survival (reviewed in (250)). The aim of those inhibitors is to block the engagement of PD-1 with PD-L1 or binding of CTLA-4 with its ligands, thus attenuating immunosuppression and induction of anti-tumor immunity (258).

Schadendorf et al. reported that melanoma patients treated with ipilimumab (targeting CTLA-4) had an estimated three-year survival of about 22 % (259), compared to a three-year survival of about 12 % after chemotherapeutic treatment (260). However, despite their standard usage as therapy for cancers, such as NSCLC (261), melanoma (262), hepatocellular (263) and microsatellite instability-high CRC (264), in some malignant diseases, response to ICI still faces hurdles and limitations (265). For example, the response rate of Hodgkin's lymphoma is above 65 %, whereas rates in other cancer types range between 10 and 30 %, indicating resistance mechanisms (266).

Generally, biomarkers for the efficacy of different ICI include PD-L1 expression, T cell infiltration, the mutational burden of the tumor, and IFN-y signaling (267), thus predicting treatment outcome. Moreover, resistance can be divided into two categories: innate resistance (patients who never responded to ICI) and acquired resistance (patients who responded in the

beginning of the treatment, but became resistant over time) (268). Related mechanisms include reduction in tumor-specific neoantigen production, altered T cell effector functions and reduced T cell infiltration, T cell exhaustion, through upregulation of other checkpoint molecules, activation of immunosuppressive cells, and defects in antigen presenting cells (reviewed in (258,265)).
In recent years combination of ICI with each other (anti-PD-1 and anti-CTLA-4) or with other cancer therapies, such as chemo- or radiotherapies have proven successful, by inducing T cell activation and antigen presentation (258,269,270).

In the end, further investigation of the interactions in the TME is needed. In detail, it is important to understand how certain pathways can be used to induce an anti-tumorigenic immune response, for example by inducing the infiltration of activated effector cells like T cells or just direct tumoricidal effects – aiming to support the response to cancer therapies, thus contributing to patient survival in the future. In this respect, the use of mediators of the TME like IL-33 and 2-AG may provide a strategy in support of a successful ICI.

1.5 Hypothesis and aims of the thesis

We hypothesized that IL-33 and the 2-AG/MGL axis would shape the TME immune cell landscape in favor of an anti-tumorigenic immune response, thus leading to reduced tumor growth.

The first aim of the thesis was to harness the ability of IL-33 and the 2-AG/MGL axis to shift the TME in our experimental models of CRC and NSCLC in favor of an anti-tumorigenic phenotype. In detail, our goal was to investigate changes in the infiltrating immune cell profile in the experimental mouse models by using flow cytometry. Additionally, we identified differences in the activation status of those cells.

As a next step, we focused on the understanding of the mediators that contributed to the changes in the TME and, therefore, influenced tumor development. Our goal was to identify differences in other soluble mediators like cytokines, chemokines, endocannabinoids, and lipids.

Lastly, we aimed to determine the direct effects of IL-33 and 2-AG on the affected immune cell populations of the TME. Thus, we used *in vitro* assays to identify their role in activation, recruitment, cytotoxicity, and viability.

2 RESULTS

The results section will summarize the following publications:

1. Melanie Kienzl, Carina Hasenoehrl, Paulina Valadez-Cosmes, Kathrin Maitz, Arailym Sarsembayeva, Eva Sturm, Akos Heinemann, Julia Kargl & Rudolf Schicho (2020) IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils, OncoImmunology, 9:1, DOI: 10.1080/2162402X.2020.1776059
2. Melanie Kienzl, Julia Kargl, Rudolf Schicho. (2020) The Immune Endocannabinoid System of the Tumor Microenvironment. International Journal of Molecular Sciences, 21(23):8929. https://doi.org/10.3390/ijms21238929
3. Melanie Kienzl, Carina Hasenoehrl, Kathrin Maitz, Arailym Sarsembayeva, Ulrike Taschler, Paulina Valadez-Cosmes, Oliver Kindler, Dusica Ristic, Sofia Raftopoulou, Ana Santiso, Thomas Baernthaler, Luka Brcic, Lisa Hahnefeld, Robert Gurke, Dominique Thomas, Gerd Geisslinger, Julia Kargl & Rudolf Schicho (2021) Monoacylglycerol lipase deficiency in the tumor microenvironment slows tumor growth in non-small cell lung cancer, OncoImmunology, 10:1, DOI: 10.1080/2162402X.2021.1965319

The TME depicts a complex environment, in which, leukocytes and other cell types, either directly via cell-cell contact or indirectly via cytokine and chemokine signaling, communicate and interact with each other and the tumor cells, contributing to tumor progression and/or suppression (271). Immune therapies, especially ICI, aiming to shape and activate an anti-tumorigenic TME, have emerged as a defense line in the fight against cancer (272). However, limitations and resistance towards ICI result in low response rates, depending on the tumor entity (266), thus highlighting the importance of understanding the complex interactions in the TME, to provide additional information and new targets for combinational treatments (265). Therefore, we aimed to add to the greater picture by identifying the role of the alarmin IL-33 and the endocannabinoid 2-AG on the TME in experimental models of CRC and NSCLC, respectively.

Summaries of results:

Ad 1.:

In my first project we investigated the role of eosinophils on IL-33-induced tumor growth reduction in experimental models of CRC (Figure 4)(1). IL-33 is a cytokine (“alarmin”), released by disrupted epi- and endothelial cells (100). Tumor-associated tissue eosinophilia was detected after IL-33 treatment of tumor-bearing mice. IL-5 and CCL24 levels were increased at the same time. We determined that eosinophils were indispensable for the IL-33-mediated reduction of tumor growth by using eosinophil-deficient ΔdblGATA-1 mice. The smaller tumor size in IL-33 treated WT mice was associated with presence of eosinophils but independent of other tumor infiltrating leukocytes (1). In line with this, IL-33-activated eosinophils showed

increased expression of CD63 and CD107a (1), both are described as degranulation markers (30,273,274). Additionally, Siglec-F as well as CD11b (1), both of which are important for the activation and transmigration of eosinophils into the tissue (275,276), were also increased. Moreover, survival and cytotoxicity of eosinophils towards tumor cells was enhanced post IL-33 treatment (1).

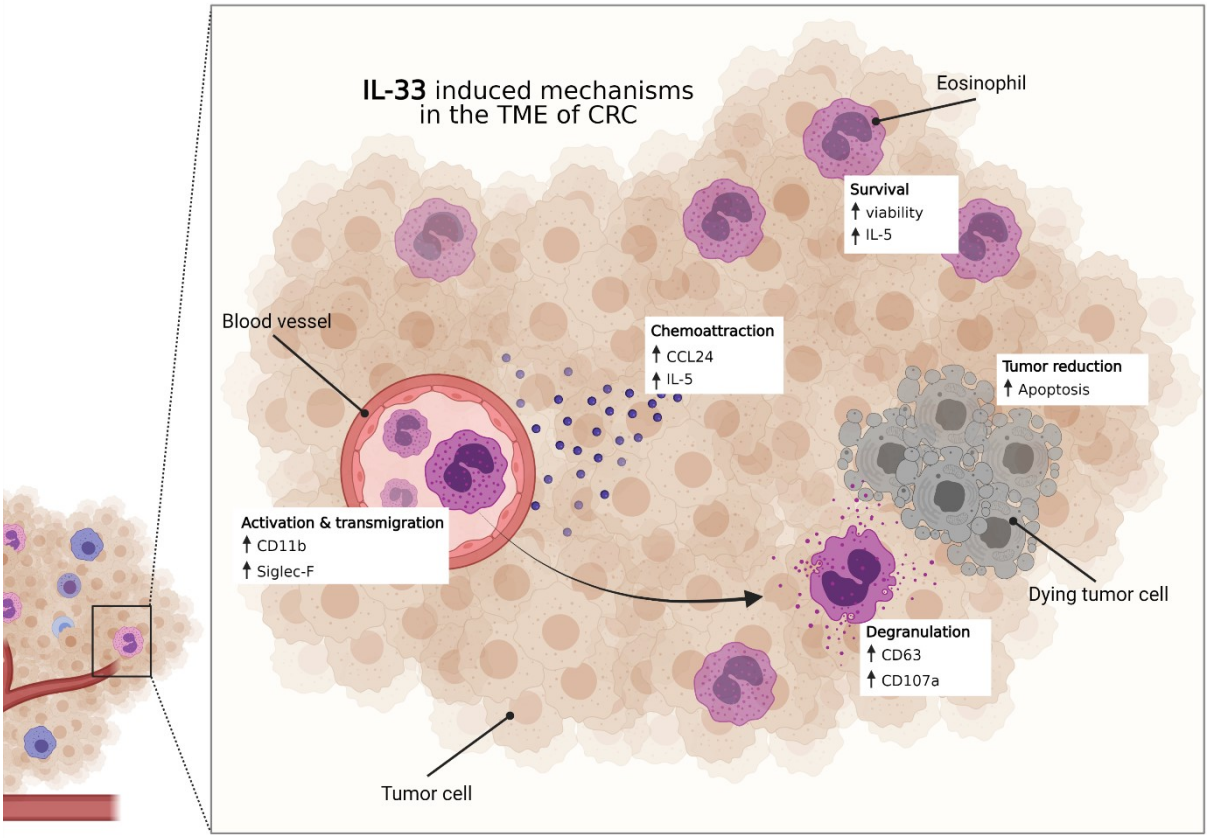

Figure 4 IL-33 mediated anti-tumorigenic changes in the TME of colorectal cancer (CRC) models are dependent on the activation of eosinophils. IL-33 treatment of CRC cell tumor bearing mice leads to induced infiltration of eosinophils into the TME and reduces tumor size. IL-33 activation of eosinophils is accompanied by increased expression of CD11b and Siglec-F, as well as CD63 and CD107a. Created with BioRender.com

Ad 2.:

As a next step, we performed a comprehensive literature review on immune cells of the TME, how they could be affected by endocannabinoids, and in particular on whether they would contribute to a pro- or anti-tumorigenic TME (2). Immune cells are an essential part of the TME, and alterations in their composition or activation contribute to either tumor development or suppression (16). The core of the ECS comprises the endocannabinoids, their receptors and endocannabinoid-synthesizing and -degrading enzymes (188). Importantly, immune cells have been described to express components of the ECS. Consequently they are able to react to endocannabinoids and, hence, the term “immune-endocannabinoid system” was introduced (277,278). The effect of endocannabinoids on leukocytes *in vitro* was already studied

thoroughly (2). However, there is lack of evidence on how the ECS contributes to changes in the TME and the tumor development, and also to the response after immunotherapy. Thus, this literature review debates on how the “immune ECS” might shape the TME and if it could have an effect on tumor growth.

Ad 3.:

Ultimately, I was focusing on identifying the role of MGL, a component of the ECS, in the TME of NSCLC (Figure 5)(3). MGL is not only an important enzyme in the lipid metabolism (90), but it is also known for the degradation of the endocannabinoid 2-AG into glycerol and FFAs (95). Therefore, MGL is representing an important player in the development of cancer, at two different ends (namely lipid and endocannabinoid metabolism). Firstly, MGL was suggested to provide building blocks for proliferating cancer cells, by degrading monoglycerides into FFAs (88). Secondly, 2-AG, which is degraded by MGL, was described to have immuno-modulatory effects on immune cells and hence potentially on the TME (2). As mentioned above, the role of the ECS, and especially of MGL, in tumor cells has been investigated in detail (197). More importantly, MGL inhibitors have been evaluated as potential cancer therapeutics (279). The role of MGL in cells of the TME, however, is less understood.

By using the MGL inhibitor JZL184 as well as MGL knock out (KO) mice we were able to show that pharmacological inhibition of MGL in cancer cells and the TME, and also genetic deletion of MGL in the TME alone reduced tumor growth in our NSCLC model (mice bearing s.c. tumors of KP cells; $Kras^{LSL\text{-}G12D}/p53^{fl/fl}$; isolated from mouse lung adenocarcinoma). Smaller tumors in MGL KO mice were accompanied by increased infiltration of anti-tumorigenic immune cell populations, such as eosinophils and $CD8^+$ T cells, which were highly activated, as measured by PD-1, IFN-γ and granzyme-B expression. MGL expression was identified in tumor cells, macrophages, and stromal cells with the help of in-situ hybridization. Interestingly, 2-AG levels were increased in tumors of MGL KO mice, and *in vitro* experiments identified 2-AG as an important mediator of eosinophil and $CD8^+$ T cell activation and chemoattraction. MGL overexpression in tumor cells resulted in increased tumor growth, but in decreased 2-AG levels as compared to control tumors (3).

Hence, our research highlights the immuno-modulatory role of MGL and, more importantly, of 2-AG in the TME of NSCLC, providing important information about the influence on eosinophil and $CD8^+$ T cell function and migration.

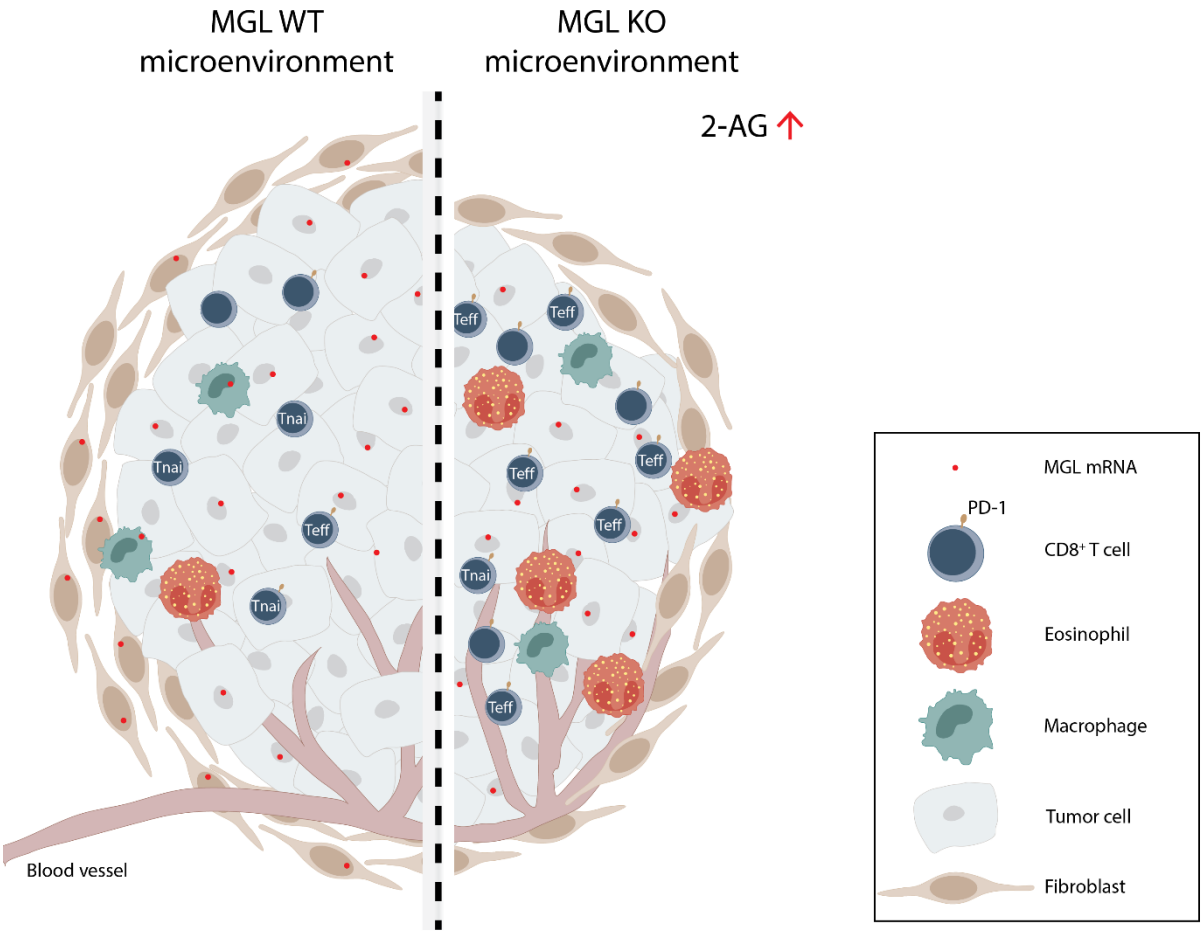

Figure 5 Alterations of the immune cell infiltrate in NSCLC in response to monoacylglycerol lipase (MGL) deficiency in the tumor microenvironment (TME). This image summarizes differences in the composition of the TME of MGL wild type and knockout mice (KO) bearing experimental NSCLC. Increased 2-arachidonoyl glycerol (2-AG) in tumors of MGL-deficient mice is accompanied by enhanced $CD8^+$ T cell and eosinophil recruitment to the tumor site. Moreover, $CD8^+$ T effector cells (Teff) and PD-1 expression by $CD8^+$ T cells are elevated. (Tnai=naïve T cells) Image reproduced from Kienzl et al. (3) under Creative Commons Attributions License.

3 DISCUSSION

The TME is a complex setting, in which stromal and immune cells communicate and interact with each other and with tumor cells to shape the TME and to influence tumor development. It directly contributes to tumor surveillance or tumor progression (16). I investigated two very different soluble mediators within my PhD studies, namely IL-33 and 2-AG. Despite their obvious differences in origin and biologic structure, both mediators were involved in an anti-tumorigenic shift of the TME, and they reduced tumor growth in tumor models of NSCLC and CRC. We observed important effects of IL-33 on eosinophil activation, survival and tumorgenicity in a model of CRC (1). 2-AG attracted our attention in a MGL deficient TME of a mouse model of NSCLC. MGL, a component of the ECS, degrades 2-AG into AA and glycerol, thus deficiency of MGL leads to increased accumulation of 2-AG in tissue (280). MGL deficiency was accompanied by an anti-tumorigenic shift in the TME and reduced tumor size. Activation of tumoricidal immune cells by 2-AG was confirmed *in vitro* (3). This finding emphasizes the ability of members of the ECS in shaping the TME and controlling cancer development. We finally contributed to an underexplored area by writing a comprehensive literature review discussing the role of endocannabinoids, their receptors, and endocannabinoid-synthesizing and -degrading enzymes in the TME (2).

In the end, this dissertation contributes to an area of research that has gained more and more importance in recent years, namely the search for effective immunotherapies, which are being used more or less successfully in the battle against cancer. Thus, the thesis provides suggestions on how to induce the immunogenicity of the TME as a potential adjuvant therapy of ICI and other immune therapies to improve disease outcome and patient survival.

3.1 **IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils.** (OncoImmunology, 9:1, DOI: 10.1080/2162402X.2020.1776059)

In my first publication we were focusing on identifying the importance of eosinophils in the TME of CRC, and especially on how IL-33 influences eosinophils in this setting.

IL-33 treatment reduces tumor growth in CRC models and is associated with eosinophil infiltration

We employed a heterotopic CRC model, using mice bearing s.c. tumors of the CRC cell line CT26 and treated them with IL-33. In line with a study by Lucarini et al. who showed reduced growth of melanoma after treatment with IL-33 (33), we detected smaller tumors in the mice treated with IL-33, when compared to the vehicle-treated mice (1). A genomic characterization

revealed, that CT26 cells are mutated in the KRAS G12D, but not in the Tp53 gene (281). These and further immunomic and transcriptomic investigations led to the suggestion that CT26-induced tumors shared molecular features with human primary CRC (281), making this model to a valuable heterotopic tool for CRC research. However, to investigate a more translational way of orthotopic tumor development, we additionally used a colitis-associated CRC model (AOM+DSS). As expected, in this model, IL-33 treatment led to improved bodyweight and reduced CRC burden compared to vehicle-treated mice (1). Increased infiltration of eosinophils was observed in both CRC models post IL-33 treatment, something that was already described in an experimental melanoma model (33). In line with this, Reichman et al. identified increased tumor burden in eosinophil-deficient ΔdblGATA-1 mice when compared to wild type littermates in the colitis-induced CRC model (25), hence, suggesting an anti-tumorigenic role of eosinophils.

The increased number of infiltrating eosinophils after the IL-33 treatment drew our attention to possible changes in mediators affecting eosinophil survival and recruitment. Thus, we used a multiplex cytokine array and observed, that in our heterotopic CRC model, the reduced tumor size was accompanied by elevated IL-5 and CCL24 levels (but not CCL11 and CCL5) (1), which are important mediators for eosinophil survival and migration, respectively (282). This observation suggested a potential involvement of these factors in the IL-33-induced eosinophilia. IL-5 not only prolongs eosinophil survival, but was also described to support eotaxin-mediated recruitment of eosinophils to the lung (283). However, increased survival of eosinophils cultured with CRC cell line-derived conditioned media was independent of IL-5 (25). Nevertheless, growth of fibrosarcoma was reduced in IL-5 transgenic mice, and this was accompanied by increased eosinophil infiltration into the tumor (284). Furthermore, CCL24 was found highly expressed in CRC biopsies (285). Loss of CCL11, on the other hand, was associated with reduced tumor growth in a model of colitis-associated CRC in a recent study by Polosukhina and colleagues (286). Nevertheless, the cell types producing above-mentioned factors and mechanisms of action are still unclear. In line with our finding, IL-33 led to the increased secretion of CCL24, but not CCL11 in an IL-33 mediated model of lung inflammation (287). Additionally, recent studies highlight a role of IL-33-activated ILC2s in IL-5 production (287) and in the stimulation of CCL24 secretion by myeloid cells (288). Whether ILC2s play a role in eosinophil recruitment and survival in our CRC cancer model, will be elucidated in future experiments.

Eosinophils directly influence tumor growth in CRC models after IL-33 treatment

Eosinophils were already described as important regulators of other infiltrating immune cells in the TME of solid tumors (76). As such, eosinophil deficiency impaired Th1 and $CD8^+$ T cell

responses resulting in defective anti-tumor immunity in CRC models (26). In line with this, they were described to induce the infiltration of $CD8^+$ T cells in experimental melanoma (31). Other reports described an influence of activated eosinophils on the infiltration of adaptive and innate immune cells, like the polarization of M1 macrophages, the maturation of DCs (289), but also a reduction of infiltrating immune suppressive myeloid cells was described (33). We used flow cytometry to investigate tumor infiltrating leukocyte populations in more detail. Unlike the publications mentioned above, we detected IL-33-induced changes only in $CD4^+$ T cells and T regs, however, no changes were observed in myeloid derived suppressor cells, $CD8^+$ T cells, or macrophages (1). The increase of tumor infiltrating T regs after IL-33 treatment is in line with recent publications in s.c. CT26 tumors and a genetic mouse model of CRC (124,143). However, in contrast to these publications, the increase in T regs in our study was not accompanied by increased tumor burden (1). Furthermore, we also detected an increased T reg frequency in IL-33-treated CT26 tumor-bearing eosinophil deficient ΔdblGATA-1 mice, without change in tumor burden, suggesting an eosinophil independent mechanism of increased T reg infiltration (1). We additionally noted a reduction in $CD8^+$ T cell infiltration after IL-33 treatment of ΔdblGATA-1 mice (1). Carretero et al. previously described a role of eosinophils in the attraction of $CD8^+$ T cells via CCL5, CXCL9 and 10 into the TME of melanoma (31), suggesting that the lack of eosinophils in our study may have caused the reduction in $CD8^+$ T cells. However, why this is only detectable post IL-33 injection of the ΔdblGATA-1 mice, is not known. The reduced monocyte infiltration that we have observed in the CT26 tumors of our IL-33-treated eosinophil deficient mice (1) was previously described in wild type mice engrafted with melanoma cells (33).

Because we observed only minor changes in the immune TME by the IL-33 treatment, we assumed that eosinophils could directly influence tumor growth in our model. As such, we investigated *in vitro* the potential of IL-33-activated eosinophils on killing CT26 tumor cells directly, hence, confirming data showing eosinophil-mediated human and mouse CRC cell killing (1,25,29) and increased apoptosis of melanoma cells when co-cultured with IL-33-activated as compared to IL-5-only treated eosinophils (30,33).

Minor involvements of immune cells in anti-tumorigenic effects of eosinophils are not uncommon. A lymphocyte independent mechanism of eosinophil-mediated anti-tumorigenic effects was investigated earlier in various experimental solid tumors by Hollande et al. (159). Furthermore, Reichman et al. observed an eosinophil-mediated anti-tumorigenic effect in colitis-associated CRC by comparing wild type and ΔdblGATA-1 mice, as well as in $APC^{min/+}$ mice crossbred with eosinophil deficient mice (25). In line with this publication we observed reduced tumor burden in colitis-associated CRC after IL-33 treatment, accompanied by

eosinophil infiltration and increased bodyweight of the mice, however, no changes in other leukocytes were detected (1).

The effects of IL-33 on colitis-associated CRC have been investigated thoroughly, but changes in eosinophils were hardly addressed. Genetic deficiency of IL-33 disrupts the IgA-microbiota axis, changing the microbiota in the gut leading to IL-1α secretion, hence, driving increased CRC development (290). Furthermore, in a model of sporadic CRC, deficiency in ST2 led to elevated CRC progression that was related to attenuation of IFN-γ expression and increased T reg infiltration (291). At this stage it must be pointed out that apart from IL-33, TNF-α and IFN-γ have been described to activate eosinophils in the TME as well. Carretero et al. investigated significantly reduced melanoma growth when mice were injected with TNF-α/IFN-γ activated eosinophils intravenously (31). Furthermore, in models of chemically and genetically induced CRC, IFN-γ-activated eosinophils were found to have anti-tumorigenic potential (25). Hence, we also measured IFN-γ in our study, however, the levels were unaltered between groups (unpublished data). Finally, Chen et al. investigated a direct effect of IL-33 on CRC human cell growth (131). Treatment with IL-33 reduced pro-proliferative and anti-apoptotic molecules leading to decreased growth (131). In our study, however, a direct influence of IL-33 on CT26 cell growth was not detected (1). In contrast to that, O'Donnell et al. reported ST2 expression by CT26 cells and attenuation of tumor growth upon ST2-shRNA treatment (132). Taken together, these studies support our work on the effect of IL-33 on CRC development.

IL-33 activates eosinophils by inducing migration, degranulation, and activation

Eosinophils were reported to express the IL-33 receptor ST2 (105), which fits with a report that IL-33 activates eosinophils in a NFκB dependent manner (157). IL-33 activation of eosinophils is followed by increased expression of molecules that facilitate transmigration into the tissue (e.g., CD11b) and promote degranulation (e.g., CD63) (30,33,78,292,293). Based on these findings, we wanted to investigate IL-33-mediated eosinophil activation in our settings. By incubating bone marrow-derived eosinophils *in vitro,* as well as by treating mice with IL-33 *in vivo*, we could confirm these findings in our study and measured increased levels of CD11b (*in vitro* and *in vivo*) and CD63 (*in vitro*) on eosinophils (1). Furthermore, Andreone et al. recently highlighted the role of CD11b on eosinophils in adhesion to tumor cells by describing the importance of the formation of a synapse between CD11b/CD18 on eosinophils and ICAM-1 on tumor cells leading to degranulation and subsequent tumor cell killing (30). Additionally to CD63, another degranulation marker, namely CD107a (274), was upregulated on eosinophils in our study, suggesting degranulation of eosinophils as a potential mechanism for the reduced tumor growth in our models (1). Interestingly, we observed increased expression of Siglec-F on tumor infiltrating eosinophils after IL-33 treatment (1). This is of high interest

because elevated Siglec-F has been related to activation and translocation of eosinophils to the airways (276). Hence, our data suggest a role of IL-33 in priming eosinophils to degranulate and migrate. In line with this, *in vitro* migration towards CCL24 was increased in IL-33- compared to IL-5-activated eosinophils, although differences in migration towards CT26 cell line-conditioned supernatant were less clear (1).

Activated eosinophils are indispensable for IL-33-mediated tumor reduction

As a next step we wanted to investigate whether presence of eosinophils was necessary for the IL-33-induced tumor reduction. Until recently, the intraperitoneal injection of anti-Siglec-F antibodies was used to deplete eosinophils (31,33). However, Knuplez et al. proposed that the application of a Siglec-F specific antibody inhibits the binding of Siglec-F with its ligands, rather than leading to eosinophil apoptosis and subsequent depletion (294). Therefore, we used CT26-tumor bearing eosinophil-deficient ΔdblGATA-1 mice and treated them with IL-33, resulting in no changes in tumor growth when compared to untreated mice, indicating that IL-33 was ineffective in the ΔdblGATA-1 mice (1). Furthermore, the repopulation of the eosinophil-deficient ΔdblGATA-1 mice with eosinophils reduced tumor growth only when the eosinophils were first activated with IL-33, but not when they were activated with IL-5 alone (1). Interestingly, IL-33-activated eosinophils were also more prone to migrate to the TME in comparison to IL-5-activated eosinophils *in vivo* (1). Taken together these results corroborate data describing reduced tumor growth of melanoma cells co-injected with IL-33-activated eosinophils (30).

We were further asking ourselves if the increased abundance of eosinophils in CT26 tumors of IL-33 treated mice could have been also due to increased survival of eosinophils (next to increased migration to the TME). Previous studies have described a role of IL-33 in extending the life span of human and mouse eosinophils (158,295). Furthermore, incubation of eosinophils with tumor cell-conditioned supernatant prolonged their survival independently of IL-5 (25). Our observations confirm these findings, as we detected increased viability after co-incubation with CT26 cells, which have been described to release IL-33 (132,296) *in vitro*. Co-incubation even enhanced survival of IL-33-stimulated eosinophils in the TME of CRC *in vivo* and *in vitro* (1).

Overall, the data propose a crucial role of eosinophils in the anti-tumorigenic effect of IL-33 in the investigated CRC models. Activation of eosinophils with IL-33 significantly diminished tumor growth. This result is probably linked to direct eosinophil-mediated killing of malignant cells rather than other anti-tumorigenic changes in the immune TME.

3.2 **The Immune Endocannabinoid System of the Tumor Microenvironment.** (International Journal of Molecular Sciences, 21(23):8929. https://doi.org/10.3390/ijms21238929)

In the beginning, the review gives a comprehensive summary about immune cells of the TME and how immune cell function contributes to a shift in the balance of immunosurveillance or tumor progression, as well as it presents data on patient survival in different tumor entities (2).

Cytokines, but also other soluble mediators, like chemokines and lipid mediators might affect the TME and, hence, influence tumor development, patient survival and response to therapy (36). Among those lipid mediators, endocannabinoids were reported to regulate immune cell functions (278). In particular, most immune cells express cannabinoid receptors, making them susceptible to endocannabinoid signaling; however they are also able the synthesize and degrade endocannabinoids themselves (2,278), creating a so-called “immune endocannabinoid system” (277). As CB_2 is expressed on diverse immune cell populations at differential levels (198), sensitivity to endocannabinoid signaling may differ considerably. Furthermore, only low or no CB_1 expression was detected in the investigated cell types (198), pointing towards a major role of CB_2 on immune cell regulation (297). It should be pointed out that cytokines, like IL-6, TNF-α and IL-1β, were reported to increase cannabinoid receptor expression in PBMCs (298), suggesting that localization and surroundings of immune cells affect CB_1 and CB_2 expression (2). As such, inflammatory molecules in the tumor microenvironment might affect cannabinoid receptor expression (2). Interestingly, increased expression of cannabinoid receptors was observed in various solid tumors (299).

Keeping the above-mentioned in mind, the consideration of an “immune-ECS” shaping the TME is likely. So far, only few studies were published describing manipulation of certain parts of the ECS and the subsequent effects on the TME as well as on tumor growth. The first example was published by Hasenöhrl et al. who reported an anti-tumorigenic shift in the TME of a chemically-induced CRC model using a GPR55-deficient mouse. This shift was reflected by decreased infiltration of MDSCs, increased infiltration of T cells, and a reduced tumor burden (300). Furthermore, Qiu et al. described no changes in T cell infiltration, but increased MDSC numbers in pancreatic tumors of 2-AG treated mice (230). Finally, MGL-deficient macrophages led to increased CRC growth in a CB_2 dependent manner (208). Collectively, these studies provide insight into the involvement of the ECS on changes in the TME and suggest that the ECS might play a role in regulating components of the TME and, as a result, in tumor development (2).
In other words, the effects of endocannabinoid-synthesis, -degradation and -signaling in immune cells is not black and white, as both, pro- and anti-tumorigenic, effects were observed

(2). This is also reflected by pro- and anti-inflammatory effects of (endo)cannabinoids on different immune cells (278). To give one example, 2-AG was reported to induce migration of B cells, DCs, and eosinophils (278). In contrast, 2-AG was also indicated to reduce expression of TNF-α, IL-6, MCP-1, adhesion molecules, and subsequently, leukocyte recruitment in a model of lung injury (301).

With regard to the complexity of (endo)cannabinoid-mediated effects on immune cells, further research is necessary to gain a better understanding of possible consequences on tumor development (2). This is of special interest, as cannabinoids are used as antiemetics for cancer patients during chemotherapy, but might be also valuable as add-on drugs in tumor therapy (302). So far, however, in contrast to preclinical models (303), unfavorable effects of *Cannabis* were reported in patients given ICI (304) associating its use during ICI with worsened outcome (305).

3.3 **Monoacylglycerol lipase deficiency in the tumor microenvironment slows tumor growth in non-small cell lung cancer.**

(OncoImmunology, 10:1, DOI: 10.1080/2162402X.2021.1965319)

In the final project of my PhD studies, we tried to fill the knowledge on the role of the ECS in the progression of lung cancer, in this case by focusing on the 2-AG/MGL axis in the TME. A pro-tumorigenic role of MGL in tumor cells has already been reported in different tumor entities (88,220,221). In lung cancer, pharmacological inhibition of MGL suppressed the development of metastasis *in vivo* and invasion of tumor cells *in vitro* (218). Similarly, the treatment with JZL184 led to reduced tumor growth in our NSCLC (KP) cell tumor model (3). On the other hand, increased spontaneous lung cancer development was observed in aged MGL KO mice (226) and MGL-overexpression suppressed colony formation in the human CRC cell line HCT116 (224), suggesting an anti-tumorigenic effect of MGL.
Despite these contradicting reports, MGL has emerged as a potential drug target for inflammatory diseases and cancer therapies (306). Thus, MGL inhibitors have been patented for the treatment of different diseases, including cancer, in the recent years (279).

Tumor growth in the NSCLC models affected by the MGL/2-AG axis in the TME

Most of the above-mentioned studies solely focused on the role of MGL in cancer cells while the role of MGL in cells of the TME is not understood. It is still unknown how MGL inhibition contributes to changes in the levels of endocannabinoids and how this influences the activation of cells in the TME. Only one study, by Xiang et al., demonstrates a anti-tumorigenic role of macrophage derived MGL in tumor progression, hence, observing a positive correlation with

patient survival (208). Thus, further research is needed for the investigation of the role of MGL in leukocytes and stromal cells infiltrating into the TME to better understand the effects of MGL inhibitors on tumor growth.

At first, to better understand the effects that might be induced by a MGL deficient TME, we used qRT-PCR, as well as in-situ-hybridization in combination with immunohistochemistry, to identify MGL expressing cells in our model. Even though basic MGL expression in tumor cells was very low *in vitro*, MGL mRNA positive tumor cells were detected *in vivo*, suggesting an upregulation of the enzyme in the mouse model *in vivo* (3). As such, MGL expression was detected in tumor and TME cells (e.g., macrophages and α-SMA$^+$ fibroblasts) of the mice, but also in human NSCLC samples (3). Interestingly, tumor infiltrating α-SMA$^+$ fibroblasts were reported to derive from tumor surrounding adipose tissue (307).

As a next step we injected MGL KO mice and their wild type littermates with KP cells to produce a s.c. tumor (3). The KP cell line we used in this publication derives from a lung adenocarcinoma in a Kras$^{LSL\text{-}G12D}$/p53$^{fl/fl}$ mouse treated with Cre recombinase-expressing adenovirus. The Cancer Genome Atlas Research Network, which presents comprehensive molecular profiling, identified 18 mutated genes in 230 lung adenocarcinoma samples before therapy. Among them, TP53 and KRAS were commonly mutated by 46 % and 33 %, respectively. (308). Further mutations included genes like EGFR and BRAF (308). Next generation sequencing of 4507 patient samples revealed 1078 patients with KRAS mutation, among which 53.5 % had additional mutations in other oncogenic pathways. Of these mutations, TP53 was the most prevalent co-occurring mutation with 39.4 % (309). Taking this into account, our KP cell line-bearing mice depict a valid model system for this malignant disease. The fact that the choice of a suitable translational model system is important was provided by Busch et al. by showing differences in leukocyte infiltrates according to the tumor driver mutation (18).

The injection of this MGL-expressing tumor cell line into the flank of a MGL KO mouse (with an MGL deficient TME), resulted in smaller tumors as compared to wild type controls (3). MGL mRNA expression positively correlated with tumor growth in our NSCLC model supporting a pro-tumorigenic role of MGL (3). Likewise, according to a study in hepatocellular carcinoma, improved patient survival was associated with low MGL expression (219). Furthermore, elevated MGL expression correlated with worse prognosis and tumor progression in lung adenocarcinoma (206). In contrast, another study pinpointed a role for ABHD6, rather than for MGL, in NSCLC progression (310). Yet, ABHD6 expression did not correlate with tumor progression in our model of NSCLC (3) and neither did ABHD12, although based on its high expression level in KP cells and tumor homogenate, a potential role in 2-AG hydrolysis in our model cannot be excluded.

MGL is the major enzyme for the hydrolysis of the endocannabinoid 2-AG (95). Thus, pharmacological inhibition or genetic deficiency of MGL was observed to elevate levels of 2-AG in mouse tissue (280). Similarly, our data showed that 2-AG levels were increased in tumors of MGL deficient mice and mice treated with the MGL inhibitor JZL184 (3), suggesting a potential role of 2-AG in reduced tumor growth in those mice. In line with this finding, anti-tumorigenic effects of 2-AG were previously described by Qiu et al. in pancreatic cancer (230), implying a potential role for the MGL/2-AG axis in cancer development. In detail, tumor growth was attenuated by 2-AG directly in a CB_1 dependent manner *in vitro* (230). Furthermore, in a work by Prüser et al. using A549 lung cancer cells, increased levels of 2-AG (after MGL inhibition) were associated with CB_1-mediated upregulation of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) in the cancer cells, leading to reduced tumor cell invasion (218). There was no association between reduction in free fatty acids (due to MGL inhibition) and reduced tumor invasion (218). Furthermore, the use of FAAH inhibitors in the human NSCLC cell line A549 increased levels of endocannabinoids, like 2-AG and AEA, leading to reduced cancer cell aggressiveness (311). In contrast to the above-mentioned studies, no direct effects of 2-AG on tumor cell proliferation or survival was observed in our study *in vitro* (most likely because CB receptor expression was quite low in cultured KP cells (unpublished observations and (3)). Apart from higher levels of 2-AG in the tumors of MGL KO vs. WT mice, we observed an increase in 2-AG levels in cancerous tissue when compared to normal tissue (3), corroborating data by Sailler et al. who show elevated 2-AG in tumor tissue (228).

MGL-deficiency in the TME induces an anti-tumorigenic TME

As no direct effect of 2-AG on tumor cell growth was observed *in vitro* (3), and owing to the fact that 2-AG is a potent mediator of immune responses (2), we performed single cell suspensions of the s.c. KP cell tumors and used flow cytometry to investigate changes in the immune cell composition of the TME. Interestingly, the TME shifted towards an anti-tumorigenic state in tumors of MGL KO mice. In fact, we observed increased infiltration of eosinophils and $CD8^+$ T cells (3). Eosinophils usually are a rare immune cell population in human NSCLC (0.3 % of all leukocytes) (312). However, they are known to have, next to pro-tumorigenic, tumoricidal effects in various solid tumors (77). Hence, the increase in eosinophil numbers in our model might have supported an anti-tumorigenic effect. When investigating the infiltrating $CD8^+$ T cells of MGL KO vs. WT mice in more detail, we discovered an increased activation status in the KO's, which manifested in elevated levels of IFN-γ and granzyme-B and a shift from naïve to effector T cells (3). Interestingly, exhausted T cells are characterized by reduced IL-2, TNF-α, IFN-γ and granzyme B secretion in an experimental model (313), thus suggesting that $CD8^+$ T cells in tumors of WT mice could have been in a state of exhaustion. However, this would need to be investigated in more detail. Clinically important and in line with

our data, decreased T effector cells (314) and aberrant regulation of T cells, including an impairment of IFN-γ production, were previously detected in NSCLC patients (315), adding translational value to our tumor model. Accordingly, an MGL deficient TME might induce tumoricidal effects by influencing the $CD8^+$ T cells. A TME-shaping effect of 2-AG was previously described in an experimental model of pancreatic cancer, but in this model, an increase in suppressive myeloid derived suppressor cells (which usually are associated with increased tumor growth) was observed, although tumor growth was reduced (230). In contrast to our findings, Xiang et al. observed that MGL inhibition in tumor associated macrophages resulted in reduced IFN-γ production and suppression of $CD8^+$ T cells in a CRC model (208). However, another study of pancreatic cancer failed to detect changes in infiltrating lymphocyte populations (230). In consideration of the aforementioned literature and our study, we assume that the influence of 2-AG and MGL deficiency on the state of the TME is dependent on the tumor entity.

In the past years, ICI became a valuable tool for NSCLC therapy (261), hence, we were interested in the checkpoint inhibitory receptor PD-1 expression changes in the TME of MGL deficient mice. We discovered increased expression of PD-1 on both $CD4^+$ and $CD8^+$ T cells (3). Fernandez-Poma et al. were comparing PD-1 positive and negative $CD8^+$ T cells in their ability to control tumor development, and they detected that only $PD\text{-}1^+$-adoptively transferred $CD8^+$ T cells managed to regulate tumor progression (316).

Overall, the changes in the infiltrating leukocyte populations in tumors with an MGL deficient TME suggest an immunosuppressive role of MGL by degrading 2-AG, which is important for recruitment, differentiation, and activation of eosinophils and $CD8^+$ T cells and, hence, for controlling the tumor development.

2-AG as an immunomodulator

To identify the mechanisms through which MGL deficiency in the TME and the subsequent increase in 2-AG might influence the TME profile, we performed migration and activation experiments *in vitro*. Using a Transwell® migration assay we could confirm that both eosinophils and $CD8^+$ T cells migrate towards 2-AG in a dose dependent manner, providing a possible pathway for increased infiltration in KP cell tumors *in vivo* (3). 2-AG, as an endocannabinoid, is a ligand for both CB_1 and CB_2 and especially the 2-AG/CB_2-axis was reported to play a role on immune cells (2). CB_1 is desensitized in MGL KO mice or mice treated with the pharmacological inhibitor JZL184 (280,317), hence, we excluded the possible participation of CB_1 in our model. The CB_2 antagonist SR144528 significantly reduced chemoattraction of $CD8^+$ T cells and eosinophils towards 2-AG in the migration experiments (3), thus corroborating data by Frei et al. suggesting that migration of eosinophils is dependent on CB_2 (238).

In contrast to eosinophils, the effector T cell phenotype of $CD8^+$ T cells was independent of CB_2 (3). Consequently, we cannot exclude additional mechanisms of 2-AG action in this tumor model. Firstly, it is known that leukocytes also express 2-AG degrading hydrolases other than MGL (207). Secondly, endocannabinoid (including 2-AG) dependent activation of the peroxisome proliferator-activated receptor γ (PPARγ) was also reported (192). PPARγ is a nuclear receptor known to antagonize the effects of the transcription factor NF-κB, therefore contributing to reduced inflammation (318). 2-AG-mediated activation of PPARγ is either dependent on cannabinoid receptor signaling or occurs independently (192,319). For instance, IL-2 is suppressed via 2-AG-mediated activation of PPARγ independently of cannabinoid receptors (320). Interestingly, activation of PPARγ on NSCLC cell lines was linked to apoptosis (321), however, we did not observe apoptosis of KP cells by 2-AG *in vitro* (3).
Thirdly, 2-AG is cleaved into AA, the precursor molecule for immunoregulatory prostaglandins and leukotrienes (322). As such, hydrolysis of 2-AG and leukotriene biosynthesis might lead to effects via leukotriene receptors as previously reported (209).

MGL overexpression in tumor cells is associated with tumor growth

As described above, MGL expression was very low in KP cell culture, but was upregulated *in vivo*, as shown by in situ hybridization (3). So, we next aimed to investigate how MGL derived from tumor cells might affect tumor progression and the TME profile. By using a lentiviral transduction method we created an MGL-overexpressing KP cell line (3). In contrast to similar proliferative behavior of MGL-overexpressing and control KP cells *in vitro*, s.c. injection of MGL-overexpressing KP cells led to increased tumor growth *in vivo* (3). In line with this finding, it was reported that overexpression in nasopharyngeal carcinoma cells induced a metastatic phenotype (323). Additionally, Nomura et al. showed high MGL expression in aggressive cancer cells, as well as a change to a more pathogenic behavior in non-aggressive cancer cells, when they overexpressed MGL (88). It is quite likely that not only TME-derived MGL, but also MGL expressed by tumor cells contributed to the tumor development in our model (3).

Distinct lipid profiles in KP cell tumor bearing mice after pharmacological and genetic MGL manipulation

At this point, it must be mentioned, that 2-AG was not the only lipid mediator showing changed levels in tumors with an MGL deficient TME. As such, increased lipid species include, the monoglyceride 2-OG, lysophospholipids like LPCs, as well as PCs and sphingomyelins (3). In some of these lipids, such as in species of PC (18:4/3:0) and LPC (18:0, 18:1, 18:2), decreased concentrations were observed, which has also been detected in lung cancer (324–326). However, whether this observation contributes to changes in tumor growth, remains a speculation and needs further research. Increased levels of endocannabinoid-like substances

(namely AEA, PEA, and OEA+VEA) were only detected in tumors of JZL184 treated mice (3). A similar increase was previously reported by applying FAAH inhibitors to A549 cells (311) suggesting potential unspecific inhibitory effects of the MGL inhibitor JZL184.

In contrast to Nomura et al. (88), no changes in FFAs were detected in tumor homogenate of MGL-overexpressing vs. control KP tumors, suggesting that, in our model, MGL overexpression in tumor cells might not contribute to the synthesis of building blocks for tumor growth or generation of pro-cancerogenic lipid mediators (3).

4 CONCLUSION

In summary, the performed experiments provide further insight into the complex interplay of cellular and soluble mediators of the TME and their influence on tumor development. We identified IL-33 and the endocannabinoid 2-AG as immune regulators of eosinophils and/or $CD8^+$ T cells. We could show that IL-33 and 2-AG may be important targets for inducing eosinophil infiltration into the tumor microenvironment, with beneficial anti-tumorigenic effects. Furthermore, both mediators enhanced eosinophil effector functions. Interestingly, eosinophilia in the peripheral blood of patients is associated with the response to ICI in melanoma (327) and breast cancer patients (328). However, the effects of IL-33 on the TME are manifold (45). Considering IL-33-induced treatment resistance in melanoma, one has to be cautious when suggesting IL-33 as an adjuvants of anti-tumor therapy (329).

MGL deficiency and subsequent 2-AG accumulation in the TME induced the recruitment and activation of $CD8^+$ T cells. Enhanced activation was reflected by PD-1, IFN-γ and granzyme B expression. Importantly, increased $CD8^+$ T cells and IFN-γ production were suggested as beneficial mechanisms for the response to ICI in NSCLC (67). Furthermore, IFN-γ signaling was proposed to induce tumoricidal effects by eosinophils (25). Our data highlights inhibition of MGL and the subsequent increase in 2-AG as a potential new adjuvant therapy to ICI in NSCLC patients.

Finally, we contributed to the understanding of the influence of IL-33 and components of the ECS on the TME and on tumor growth. These findings might aid in the development of drugs that could be used in combination with immunotherapies to overcome resistance mechanisms. However, further research is needed to fully understand the consequences of targeting the proposed mediators in cancer patients.

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ORIGINAL RESEARCH

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IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils

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ABSTRACT

In many types of cancer, presence of eosinophils in tumors correlate with an improved disease outcome. In line with this, activated eosinophils have been shown to reduce tumor growth in colorectal cancer (CRC). Interleukin (IL)-33 has recently emerged as a cytokine that is able to inhibit the development of tumors through eosinophils and other cells of the tumor microenvironment thereby positively influencing disease progress. Here, we asked whether eosinophils are involved in the effects of IL-33 on tumor growth in CRC.

In models of CT26 cell engraftment and colitis-associated CRC, tumor growth was reduced after IL-33 treatment. The growth reduction was absent in eosinophil-deficient ΔdblGATA-1 mice but was restored by adoptive transfer of *ex vivo*-activated eosinophils indicating that the antitumor effect of IL-33 depends on the presence of eosinophils. *In vitro*, IL-33 increased the expression of markers of activation and homing in eosinophils, such as CD11b and Siglec-F, and the degranulation markers CD63 and CD107a. Increased expression of Siglec-F, CD11b and CD107a was also seen *in vivo* in eosinophils after IL-33 treatment. Viability and cytotoxic potential of eosinophils and their migration properties toward CCL24 were enhanced indicating direct effects of IL-33 on eosinophils. IL-33 treatment led to increased levels of IL-5 and CCL24 in tumors.

Our data show that the presence of eosinophils is mandatory for IL-33-induced tumor reduction in models of CRC and that the mechanisms include eosinophil recruitment, activation and degranulation. Our findings also emphasize the potential use of IL-33 as an adjuvants in CRC immunotherapy.

Abbreviations: AOM: azoxymethane; bmRPMI: bone marrow RPMI; CRC: colorectal cancer; CFSE: carboxyfluorescein succinimidyl ester; DSS: dextran sulfate sodium; EPX: eosinophil peroxidase; INF-γ: interferon gamma; ILC: innate lymphoid cell; IL-33: interleukin-33; IL-5: interleukin-5; MDSC: myeloid derived suppressor cells; NK cells: natural killer cells; P/S: penicillin/streptomycin; rm: recombinant mouse; T regs: regulatory T cells; TATE: tumor associated tissue eosinophilia; TNF-α: tumor necrosis factor alpha

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Introduction

Eosinophils have been originally regarded as effector cells in allergic diseases and as a defense against helminths. Over the years, however, presence of eosinophils has been consistently reported in tumor tissue (TATE, tumor associated tissue eosinophilia). Many of these reports demonstrate that tissue eosinophilia correlate with a better disease prognosis.[1] Also in studies of human colorectal cancer (CRC), increased numbers of eosinophils in tumors have been shown to serve as an independent favorable factor correlating with better prognosis and longer patient survival.[2–5] High counts of eosinophils, particularly in tumor stroma, were associated with a decreased risk for CRC death.[6]

A recent study has revealed that activated eosinophils play an important part in the reduction of tumor growth of intestinal cancer indicating that the presence of eosinophils in cancers of the gastrointestinal tract may oppose neoplasia.[7] On the other hand, in a model of oral carcinogenesis, eosinophil-deficient ΔdblGATA-1 mice were less affected by tumor growth than the wild types,[8] suggesting that the pro-/anti-tumorigenic role of eosinophils in tumor development is dependent on the type of cancer (recently reviewed in[9]). Eosinophils are able to destroy cancer cells through degranulation and the release of cytotoxic granules which contain major basic protein, eosinophil cationic protein, and eosinophil peroxidase (EPX).[1,10] Granzyme A and tumor necrosis factor alpha (TNF-α) have been also identified as eosinophil-derived tumoricidal mediators causing apoptosis and necrosis of Colo-205 colon carcinoma cells, a process that requires CD11a/CD18-dependent adhesion of eosinophils to cancer cells.[10] In general, eosinophils have been described as effector cells of immune surveillance that release cytokines to modulate functions of other leukocytes while also expressing receptors for cytokines, chemokines, growth factors, lipids and adhesion molecules to integrate inflammatory signals.[11] As to their role in solid tumors, eosinophils are thought to orchestrate a program to reduce tumor growth with the help of other leukocytes such as $CD8^+$ T cells that are attracted to the tumor site via eosinophil-produced chemokines.[12]

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A newly emerged cytokine that is able to influence tumor development via eosinophil activation is interleukin (IL)-33.[13] Although thought to be mostly pro-tumorigenic in many types of cancer[14-16] including CRC,[17–24] IL-33 was recently shown to reduce tumor growth in skin cancer and interestingly also in CRC models.[25–27] IL-33 is a member of the IL-1 family and normally released from damaged epithelial and endothelial barrier cells acting as an alarmin.[14] In this function, it can activate leukocytes, such as mast cells, eosinophils, Th2, regulatory T (Treg), $CD8^+$ and natural killer (NK) cells, and also innate lymphoid cells (e.g. ILC2).[14] IL-33 can influence immune cells of the tumor microenvironment by inducing IL-12-dependent Th1 cell differentiation in human and mouse $CD4^+$ T cells,[28] a cell type known to be associated with a good prognosis in CRC.[29] In fact, IL-33 has been proposed as a vaccine adjuvants in cancer immunotherapy because it can cause antigen-specific polyfunctional $CD4^+$ and $CD8^+$ T-cell responses.[30] The addition of a danger signal (alarmin) like IL-33 may help to overcome tumor immune tolerance, a major obstacle in cancer immunotherapy.[31] On the other hand, transgenic mice expressing IL-33 in intestinal epithelial cells and crossed with $APC^{min/+}$ mice showed increased tumor growth and higher expansion of Tregs than their littermates.[19] It has been, therefore, suggested that IL-33 expressed in tumor cells may promote immune responses in $CD8^+$ T cells and NK cells while IL-33 from tumor stroma favors tumor growth via immune suppression through Tregs and MDSCs,[31] important findings that need to be considered before using IL-33 in immunotherapy. Recent reviews on the role of IL-33/ST2 (IL-33 receptor) in tumorigenesis have discussed in more detail how remodeling of the tumor microenvironment by IL-33 may either promote or reduce tumor growth.[16,32]

Two reports have now shown that IL-33 directly activates eosinophils and reduces pulmonary metastasis and growth of melanoma.[13,25] As for CRC however, it is still unclear whether eosinophils play a role in IL-33-induced reduction of tumor growth. In the current study, we, therefore, used two *in vivo* models to investigate whether eosinophils are involved in the effects of IL-33 on tumor growth in CRC. We can report that IL-33 is a potent cytokine that diminishes tumor growth in both models. Using ΔdblGATA-1 mice, we further show that the effects of IL-33-induced tumor reduction are dependent on the presence of eosinophils.

Methods

Mice and cell line

All animal experiments were performed in the animal facilities of the Medical University of Graz. Experimental protocols were approved by the Austrian Federal Ministry of Science and Research (animal license numbers: BMWF-66.010/0076-V/3b/2018 and BMBWF-66.010/0041-V/3b/2018. BALB/c mice were either bred in house or obtained from Charles River). ΔdblGATA-1 mice were initially obtained from Dr. Helene Rosenberg (NIH, Bethesda, MD, USA) and bred in our facilities. Male CD-1 mice were obtained from Charles River. CByJ.B6-Tg(UBC-GFP)30Scha/J mice were purchased from Jackson Laboratory and bred in our facilities.

The colon carcinoma cell line CT26 was obtained from ATCC (ATCC® CRL-2638™) and maintained in RPMI with 10% FBS (Life Technologies; # 21875–091 and # 10270106) and 1% penicillin/streptomycin (P/S; PAA Laboratories; P06-07100) at 37°C and 5% CO_2 in a humidified atmosphere.

Murine tumor models and IL-33 treatment

For the heterotopic CRC tumor engraftment model, 1×10^5 CT26 cells were injected subcutaneously (s.c.) into the flank of 8–12 week old BALB/c or ΔdblGATA-1 mice. When tumors were palpable (after ~1 week), mice were treated intraperitoneally (i.p.) with 0.4 µg recombinant mouse (rm)IL-33 (Biolegend; # 580506) or PBS (as a negative control) every other day six times in total.[25] Tumor progression was monitored during the course of the experiment. Mice were sacrificed 24 hrs after the last injection, and tumors were collected. Tumors were weighed, and then measured with a caliper. The tumor volume was calculated by using the formula (v = length x width x height x π/6).

Colitis-associated CRC was induced in CD-1 mice as described before.[33] In brief, mice were first injected i.p. with 10 mg/kg of azoxymethane (AOM; Sigma-Aldrich; A5486). 2% of dextran sulfate sodium (DSS; MP Biomedicals; # 216011090) was then added to their drinking water from day 7–13 and 28–34. IL-33 treatment was started seven days after the last bout of DSS and applied i.p. at 1 µg/mouse two to four times per week. On day 99, mice were sacrificed, the colon was removed and opened longitudinally. Tumors were counted and tumor areas were measured with a caliper. Tumors were excised and kept in RPMI on ice until further use (or fixed in 10% Roti®-Histofix; Carl Roth; A146.5).

Single cell suspensions

Single cell suspensions of s.c. tumors were prepared as previously described.[34] Tumors were cut in small pieces and digested with collagenase (CLS-1; 4.5 U/ml; Worthington) and DNase I (160 mU/ml; Worthington; LS002006) for 25 min at 37°C while rotating at 1000 rpm. Digestion was only interrupted once by shortly vortexing samples. Thereafter, tissue was passed through a 40 µm strainer and washed with PBS + 2% FBS.

Dissected tumors of the colon from the AOM+DSS model were cut in small pieces and digested in PBS (containing Ca^{++} and Mg^{++}), supplemented with 5% FBS, 1 mg/ml collagenase A (Roche; #10103586001) and 160 mU/ml DNase I for 40 min at 37°C while shaking at 1000 rpm. Tissue was afterward passed through a 100 µm strainer and washed with PBS + 2% FBS. After washing, cells were counted and used for antibody staining.[35]

Flow cytometric phenotyping of immune cell populations

Prior to immunostaining,[34] single cell suspensions were incubated with 1 µg TruStain FcX™ (Biolegend; # 156604). Staining

was then performed for 30 min on ice (protected from light) with the following antibodies: CD45-AF700 (# 103128), CD45-BV785 (# 103149), Ly6 C-APC (# 128015), Ly6 G-PE/Dazzle594 (# 127648), CD11 c-BV605 (# 117334), CD8-PerCPCy5.5 (# 100734), CD63-PE (# 143903), CD107a-BV421 (# 121618) (all antibodies from Biolegend), CD11b-BUV737 (# 612801), CD11b-PECy7 (# 561098), F4/80-BUV395 (# 565614), CD3-BUV395 (# 563565), CD4-BUV496 (# 564667), Siglec-F-PE (# 562068) (all antibodies from BD Biosciences) and FoxP3-PE (eBioscience; # 12–5773-82). For nuclear antigen staining, cells were permeabilized with Transcription Factor Buffer Set (BD Biosciences, # 562574) prior to staining procedures. Dead cells were excluded with Fixable Viability Dye (FVD) eFluor™ 780 (eBioscience; #65-0865-14) according to the manufacturer's protocol. Stained cells were washed, fixed with IC Fixation Buffer (eBioscience; # 00–8222-49), and stored at 4°C until analysis. Samples were analyzed on a BD LSRFortessa™ flow cytometer with BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). Per sample, >2 x 10^5 events were recorded. Data were compensated and analyzed with FlowJo software (TreeStar, Ashland, OR, USA). Gates were defined by fluorescence-minus-one samples. See *Supplementary* Fig. 1 for gating strategies.

Immunohistochemistry/histochemical staining

Paraffin-embedded sections of mouse tumors were cut (5 µm) and deparaffinized. For immunohistochemistry, sections were microwaved for 2 x 5-min cycles in 10 mM citrate buffer, and processed by ABC method according to the manufacturer's protocol (Vectastain ABC kit; Vector Labs; PK-6101). Sections were then incubated with biotinylated mouse anti-EPX antibody (clone MM25-82.2.1; 5 µg/ml; antibody kindly donated by Dr. Elizabeth Jacobsen) and visualized with 3–3´-diaminobenzidine (DAB; Vector Labs; SK-4100). Images were taken with a high resolution digital camera (Olympus UC90) and analyzed by Olympus cellSense Standard 1.17 imaging software (Olympus, Vienna, Austria). Contrast, brightness and color balance of images were adjusted using Corel Photo Paint® (Corel Corp.). For histochemical staining of eosinophils, Sirius Red (Direct Red 80®, Sigma-Aldrich; # 365548) was used in deparaffinized sections. Sirius Red-stained tissue was counterstained with Gill's hematoxylin II (Carl Roth; T864.2).

Protein extraction and cytokine analysis

Snap frozen tumor tissue was lysed in RIPA buffer (Thermo Fisher; # 89900) and homogenized in a Percellys 24 homogenizer (VWR, Vienna, Austria) by using ceramic beads (VWR; #432-0356). Subsequently, the protein lysate was centrifuged at 14,000 rpm for 10 min (4°C) before protein concentration was determined by a Pierce™ BCA Protein Assay Kit (Thermo Fisher; # 23227) according to the manufacturer's protocol. Cytokine expression was evaluated by ProcartaPlex Multiplex Immunoassay (affymetrix eBioscience; PPX-13). The CCL24 ELISA was performed according the manufacturer's protocol (Thermo Fisher; EMCCL24).

Differentiation and activation of bone marrow derived eosinophils

Bone marrow was isolated from BALB/c and CByJ.B6-Tg(UBC-GFP)30Scha/J mice and eosinophils were differentiated as previously published.[36] In brief, erythrocytes in bone marrow were lysed using ddH_2O followed by neutralization with 10xPBS. The cells were cultured in bmRPMI, i.e. RPMI + 20% HyClone FBS (GE Healthcare; # 10309433), 1% P/S, 25 mM HEPES (Thermo Fisher; # 15630–080), 1 x non-essential amino acids (Thermo Fisher; # 11140–035), 1 mM sodium pyruvate (Thermo Fisher; # 11360–039) and 50 µM beta-mercaptoethanol (Sigma-Aldrich; M3148) supplemented with 100 ng/ml stem cell factor (PreproTech; # 250–03) and 100 ng/ml FLT3L (PreproTech; # 250–31 L). On day four, medium was changed to bmRPMI supplemented with 10 ng/ml IL-5 (Bio-Techne; # 405-Ml-005) only, to differentiate progenitors into eosinophils. Fresh medium was added every second day. On day 8 and 12, cells were transferred into a new flask. On day 13, 100 ng/ml IL-33 was added to half of the eosinophils for activation (referred to as IL-33 Eos) while the other half was kept in bmRPMI/IL-5 alone which served as control (referred to as IL-5 Eos). Before use, eosinophils were washed twice in PBS to remove IL-33 and IL-5. Purity and viability was checked using flow cytometry. For intravenous (i.v.) injections, IL-5 Eos and IL-33 Eos were either stained with the fluorescent markers CFSE or eFluor™ 450 (eBioscience; #65-0842-85 and #65-0820-84) according to the manufacturer's protocol) or used unstained, at a concentration of 5–10 × 10^6 cells/200 µl PBS per recipient mouse.

Eosinophil migration assay

Eosinophil migration assays were performed using 5 µm transwell plates (Corning; CLS3387-8EA) as previously described by us.[37] In brief, 1 × 10^5 IL-33 Eos (or IL-5 Eos) were put in the upper well. Supernatant from CT26 cell lines (conditioned for four days) and unconditioned medium was used for chemoattraction in the lower well. Recombinant CCL24 (eotaxin-2; Immunotools; # 11344174) was used as a positive control for eosinophil migration at the indicated concentrations. Eosinophils that migrated to the lower well were enumerated on a FACS Canto (BD Biosciences) as described previously.[38]

Cytotoxicity and viability assay

Eosinophil cytotoxicity assays were carried out as described before.[7] IL-5 Eos (or IL-33 Eos) were co-incubated with CT26 cells (4x10^4) in a 96-well plate at different ratios for eosinophils to tumor cells (E:T). To be able to differentiate between eosinophils and CT26 cells, we either used GFP^+ eosinophils from CByJ.B6-Tg(UBC-GFP)30Scha/J mice (Jackson Laboratory) or we stained CT26 cells with eFluor™ 450 (500 nM). After 6, 7 or 24 hrs, eosinophils were collected, and CT26 tumor cells were detached using trypsin/EDTA (PAN Biotech; P10-023100). Cells were stained with Annexin-V (BD Biosciences;

5566547; according to the manufacturer's protocol) or Zombie NIR™ Fixable viability dye (Biolegend; # 423105). The percentage of dead CT26 cells (or living GFP$^+$ eosinophils) was then analyzed as Annexin-V or Zombie NIR™ Fixable viability dye positive or negative cells using flow cytometry.

Statistical analysis

Statistical analyses for *in vitro* and *in vivo* experiments was performed using GraphPad Prism 6.1 (GraphPad Software). Significant differences between two experimental groups were determined using unpaired or paired student's *t*-tests, multiple *t*-tests or two-way ANOVA with the Sidak's post hoc test for corrections of multiple comparisons. For comparison of three groups, one-way ANOVA was used with the indicated post hoc test for corrections of multiple comparisons.

Results

IL-33 increases infiltration of eosinophils into tumors of CRC models and causes reduction of tumor growth

In the CT26 cell engraftment tumor model with BALB/c mice (Figure 1a), tumor growth was decelerated by treatment with IL-33 as compared to vehicle treatment (control) (Figure 1b). Weights and volumes of tumors, measured at the end of the experiment, were reduced by more than 50% (Figure 1c). Flow cytometric analysis of tumors revealed increased infiltration of eosinophils, CD4$^+$ T cells and Tregs after IL-33 vs. vehicle treatment (control) (Figure 1d). In Sirius Red stainings from tumors of IL-33-treated mice, eosinophils appeared less granulated than those from tumors of vehicle-treated (control) animals (inserts at the right upper corners of the images) suggesting that degranulation of eosinophils has occurred *in vivo* (Figure 1e, also see *Supplementary* Figure 2 showing anti-EPX antibody-stained eosinophils). We also detected increased levels of IL-5 and CCL24 in tumors of IL-33-treated mice, whereas levels of CCL11 (eotaxin-1) and CCL5 did not differ between the two groups (figure 1f).

In the AOM+DSS colitis-associated CRC model (Figure 1g), IL-33 was applied one week after the last cycle of DSS in order not to interfere with the (inflammatory) effect of DSS. Also here, treatment with IL-33 reduced tumor growth, as evaluated by tumor number and tumor area (Figure 1h). By the end of the experiment, IL-33-treated animals had also gained more weight than the ones treated with vehicle (control) (see fold bodyweight in Figure 1i). Our flow cytometric data showed that the number of eosinophils was significantly increased in tumors by IL-33 treatment (Figure 1j). We did not detect differences in the other investigated leukocytes between the two groups (Figure 1j). Sirius Red stainings of eosinophils in colonic tumors of the AOM+DSS colitis-associated CRC model are shown in Figure 1k. Eosinophils can be seen throughout the tumor tissue, typically located in adenomatous crypts.

IL-33 increases markers of activation, homing and degranulation in eosinophils

To investigate whether IL-33 causes anti-tumorigenic effects directly via eosinophils, we first differentiated bone marrow eosinophils from BALB/c mice and added 100 ng/ml of IL-33 for 20 hrs to the culture medium (IL-33 Eos). The other half of the cells was kept in normal IL-5-supplemented culture medium (IL-5 Eos) and served as a control. *In vitro*, incubation with IL-33 increased the expression of markers for activation and homing such as CD11b[13,39] and Siglec-F,[40] and markers of degranulation, such as CD63[13,41] and CD107a,[42] in eosinophils (Figure 2a).

Eosinophils were also investigated in tumors of the *in vivo* models. In s.c. engrafted mice, eosinophils showed increased expression of Siglec-F, CD11b and CD107a post IL-33 treatment (Figure 2b). In eosinophils from tumors of IL-33-treated AOM +DSS mice, Siglec-F but not CD107a ($p = .0526$) was significantly higher than in vehicle-treated (control) animals. No change for CD11b was detected in the AOM+DSS+IL-33 mice vs. vehicle (control) (Figure 2c). Our data, therefore, indicate that IL-33 leads to increased expression of molecules involved in activation, homing and degranulation of eosinophils *in vitro* and *in vivo*.

IL-33 enhances migration and viability properties of eosinophils

Because infiltration of eosinophils into tumors was increased in response to IL-33 treatment we next investigated whether migration of eosinophils toward CT26 cell-conditioned supernatant and CCL24 could be modulated by IL-33 pre-treatment. By use of trans-well migration assays, we observed that activation with IL-33 enhanced the migration of bone marrow-derived eosinophils toward CCL24 as compared to IL-5 Eos, and also slightly (though not significantly; $p = .062$) toward CT26 cell-conditioned medium (Figure 3a).

IL-33 is also an important cytokine for eosinophil survival[43] but whether the tumor microenvironment influences this property is unknown. We carried out *in vitro* experiments and noticed increased survival of IL-33 Eos when they were co-incubated at different ratios with CT26 cells (or medium only) indicating that CT26-conditioned medium enhanced eosinophil viability (Figure 3b). We then investigated the role of IL-33 in eosinophil migration and survival *in vivo* using eosinophil-deficient ΔdblGATA-1 mice engrafted s.c. with CT26 cells. After two weeks, we injected IL-33 Eos (eFluor™ 450-stained) and IL-5 Eos (CFSE-stained) i.v. into the mice (in a 1:1 ratio/mouse) and monitored the distribution and viability of the injected eosinophils 24 and 96 hrs post-injection. A representative flow plot for the identification of the i.v. injected eosinophils, gated as live/CD45$^+$/CD11b$^+$ eFluor™ 450$^+$ (IL-33 Eos) and CFSE$^+$ (IL-5 Eos), is shown in Figure 3c. Prolonged survival of IL-33 Eos (as compared to IL-5 Eos) could be detected for up to 96 hrs post eosinophil injection in the blood of ΔdblGATA-1 mice (Figure 3d). CT26 cell-engrafted tumors of ΔdblGATA-1 mice showed an increased number of IL-33 Eos after 24 hours (though not significantly different to IL-5 Eos; Figure 3e). However, significantly more IL-33 Eos (vs. IL-5 Eos) were seen 96 hrs after eosinophil injection (Figure 3e). Altogether,

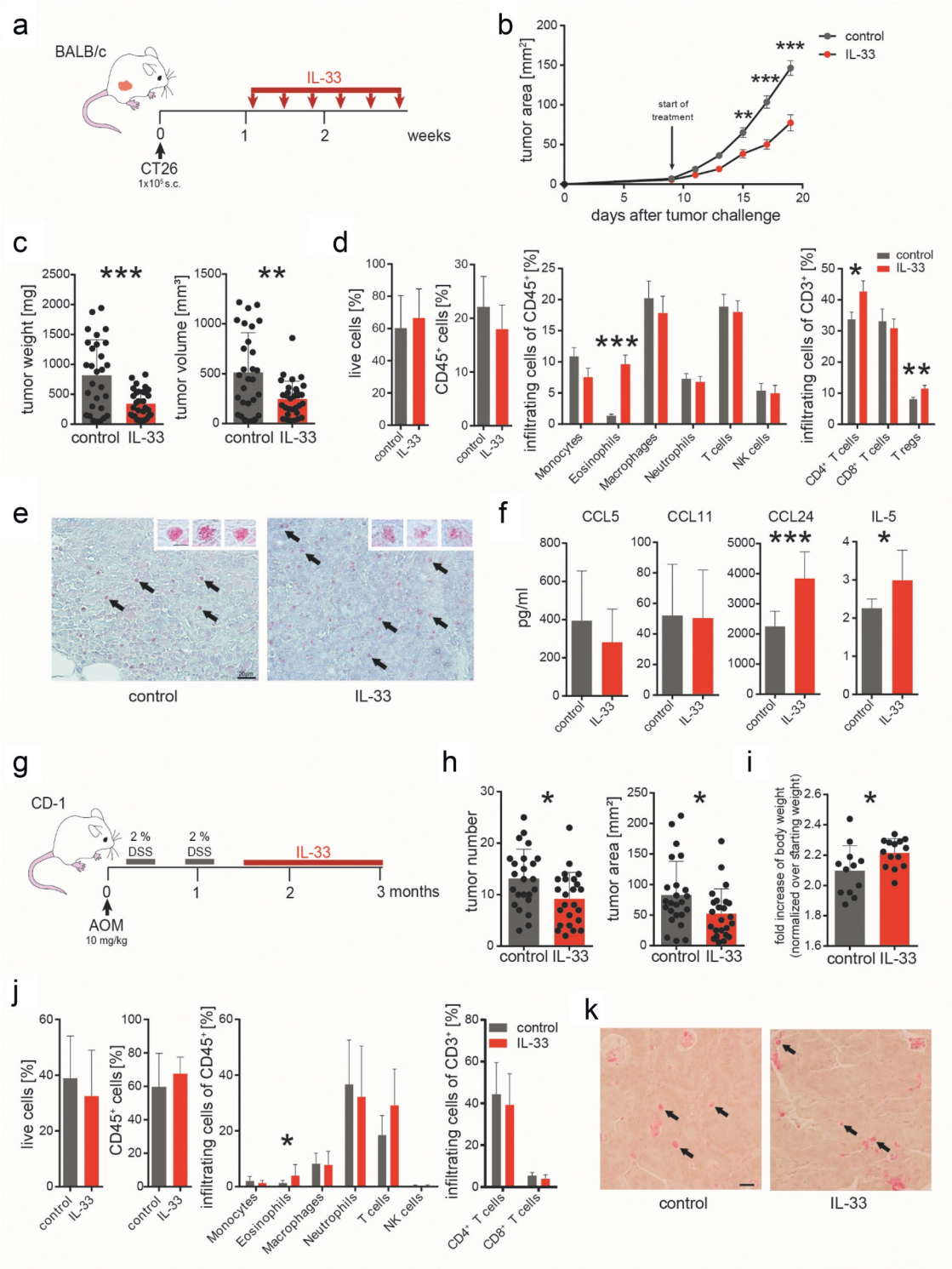

Figure 1. IL-33 causes eosinophil infiltration into tumors and reduced tumor growth in models of CRC. (a) BALB/c mice were subcutaneously (s.c.) engrafted with 1×10^5 CT26 colon cancer cells (time point 0). IL-33 treatment (0.4 µg/mouse i.p. every other day for six times) was started when tumors were palpable (after ~1 week). (b) Tumor development was monitored during the course of the treatment. Data indicate mean values ± SEM from 3 independent experiments. n = 23–25. (c) One day after the last IL-33 injection, mice were sacrificed and tumor weight and volume was measured *ex vivo*. Data were pooled from three independent experiments. n ≥ 29. (d) Flow cytometric analysis of single cell suspensions from s.c. tumors. Data were pooled from 3 independent experiments; n = 13–21. (e) Sirius Red staining of eosinophils in s.c. tumors of IL-33- and vehicle-treated (control) mice (representative images from n = 3/group; calibration bar: 20 µm). Arrows denote examples of Sirius Red-stained eosinophils. Inserts in the right upper corners show enlarged Sirius Red-stained eosinophils with granules easily detectable (calibration bar: 5 µm). (f) Immunoassay of lysed tumor tissue shows significant differences in the levels of IL-5 and CCL24 between IL-33- and vehicle-treated (control) mice; n = 7–10. Data indicate mean values ± SD. (g) Schematic presentation of the AOM+DSS-induced CRC model as performed in CD-1 mice treated with 1 µg IL-33/mouse i.p. 2–4 times per week. (h) Tumor number and area were measured in each colon showing a reduction in tumor growth in the AOM+DSS+IL-33 treated mice; n = 24–27. (i) Body weights (normalized to the starting weight) of IL-33- and vehicle-treated (control) mice. Data represent one of two independent experiments; n = 12–14. (j) Flow cytometric analysis of tumor infiltrated leukocytes. Data indicate mean values ± SD; n = 7–18. (k) Sirius Red staining of eosinophils in tumor tissue of AOM+DSS+IL-33- and vehicle-treated (control) mice (calibration bar: 20 µm; representative image from n = 3–4/group). Arrows denote representative examples of Sirius Red-stained eosinophils. Statistical differences were assessed by using two-way ANOVA with Sidak's post hoc test and unpaired student's *t*-test. *p < .05; **p < .01; ***p < .001.

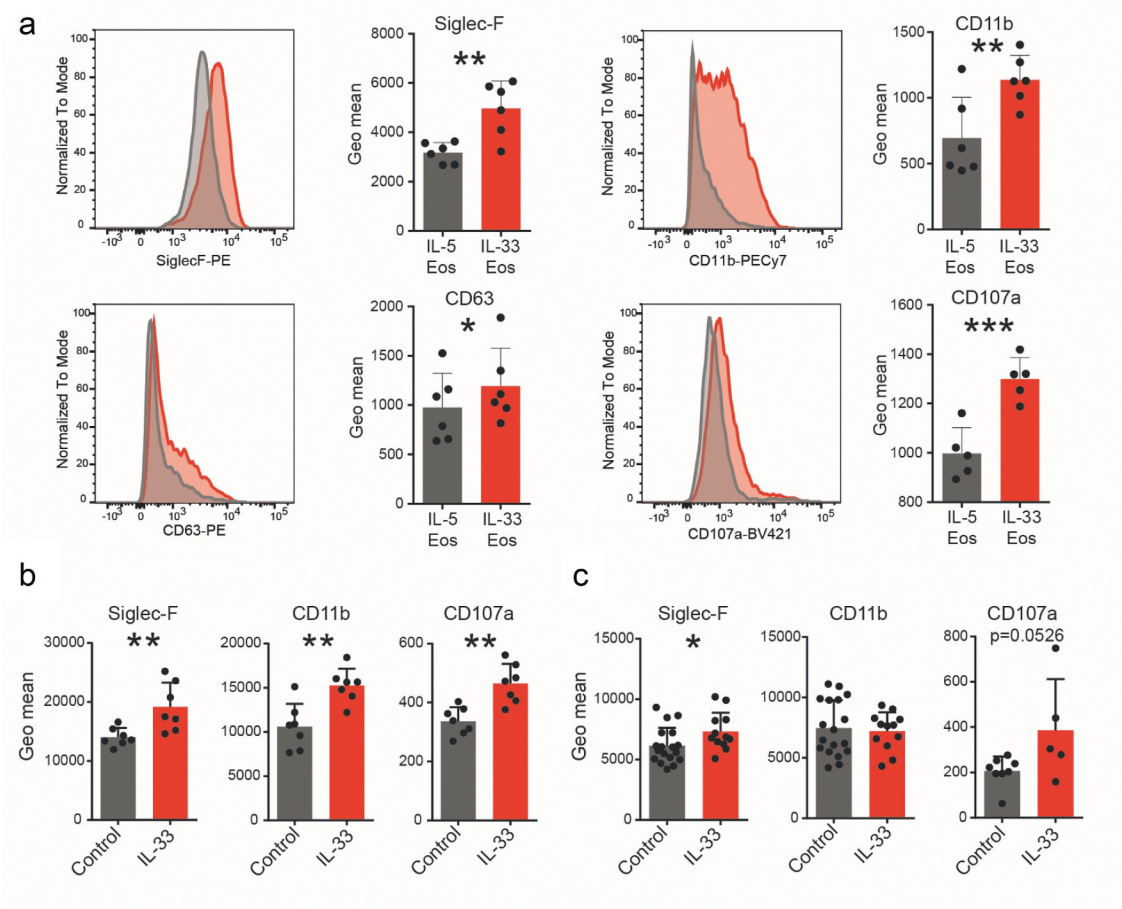

Figure 2. Expression of markers for activation, homing and degranulation. Bone marrow-derived eosinophils were activated with IL-33 (IL-33 Eos) for 20 hrs or incubated with IL-5 (IL-5 Eos) as a control, and marker expression was evaluated using flow cytometry. (a) Histograms show representative flow cytometry experiments. Next to the histograms, the graphs show paired data of two treatments (IL-5 and IL-33). Data represent one of two independent experiments; n ≥ 5. (b) Expression of markers for homing and activation (Siglec-F and CD11b) and for degranulation (CD107a) were evaluated in tumor infiltrating eosinophils from IL-33- and vehicle-treated BALB/c mice (control) (s.c. tumor model; n = 7) and from (c) IL-33- and vehicle-treated (control) AOM+DSS mice (data pooled from two independent experiments; n = 12–18. Statistical differences were assessed by using paired and unpaired student's *t*-test. *p < .05; **p < .01; ***p < .001.

these findings suggest that IL-33 primes eosinophils for migration and prolonged survival not only *in vitro* but also *in vivo*.

IL-33-induced reduction of tumor growth depends on the presence of eosinophils

To test whether the anti-tumorigenic effect of IL-33 depends on the presence of eosinophils we used eosinophil deficient ΔdblGATA-1 mice. After s.c. engraftments with CT26 cells, mice were treated with 0.4 µg rmIL-33 every other day i.p. as described before (BALB/c mice received the same treatment) (Figure 4a). There was no difference in the time course of tumor development between IL-33- and vehicle-treated (control) ΔdblGATA-1 mice (Figure 4b). Similarly, no differences in tumor weight and volume were observed in ΔdblGATA-1 mice (Figure 4c) (we also failed to see differences in the proliferation of IL-33- and vehicle-incubated CT26 cells *in vitro*; see *Supplementary* Fig. 3), while treatment of BALB/c mice confirmed our observation that IL-33 reduces tumor growth (Figure 4b,c). Flow cytometric analysis of tumor single cell suspensions showed a reduction in $CD45^+$ cells, monocytes, $CD8^+$ T cells and an increase in Tregs after treatment with IL-33 vs. vehicle in ΔdblGATA-1 mice (Figure 4d). There was a marked increase of eosinophils and a slight, though not significant, increase of Tregs (*p* value =.0616) in IL-33 vs. vehicle-treated BALB/c mice (Figure 4d).

Our data suggest that eosinophils are needed for IL-33-dependent reduction of tumor growth.

Activation of eosinophils by IL-33 enhances the reduction of tumor growth

To confirm that eosinophils are indispensable for the IL-33-induced reduction in tumor growth we argued that repopulation of ΔdblGATA-1 mice with eosinophils would restore the tumor growth-reducing effect. IL-33 Eos or IL-5 Eos were, therefore, injected twice a week i.v. into ΔdblGATA-1 mice bearing s.c. CT26 tumors (Figure 4e). Differentiation and viability of IL-33 Eos and IL-5 Eos were about 90% at the time of i.v. injections (see *Supplementary* Fig. 4). Tumors were slightly, though not significantly, reduced in mice repopulated with IL-5 Eos as compared to mice that did not receive eosinophils (PBS only) (Figure 4f). However, when IL-33 Eos were injected i.v. into ΔdblGATA-1 mice, tumor volumes and weights were significantly reduced (Figure 4g). The percentage of IL-33 Eos in tumors was clearly increased (vs. IL-5 Eos) (Figure 4h).

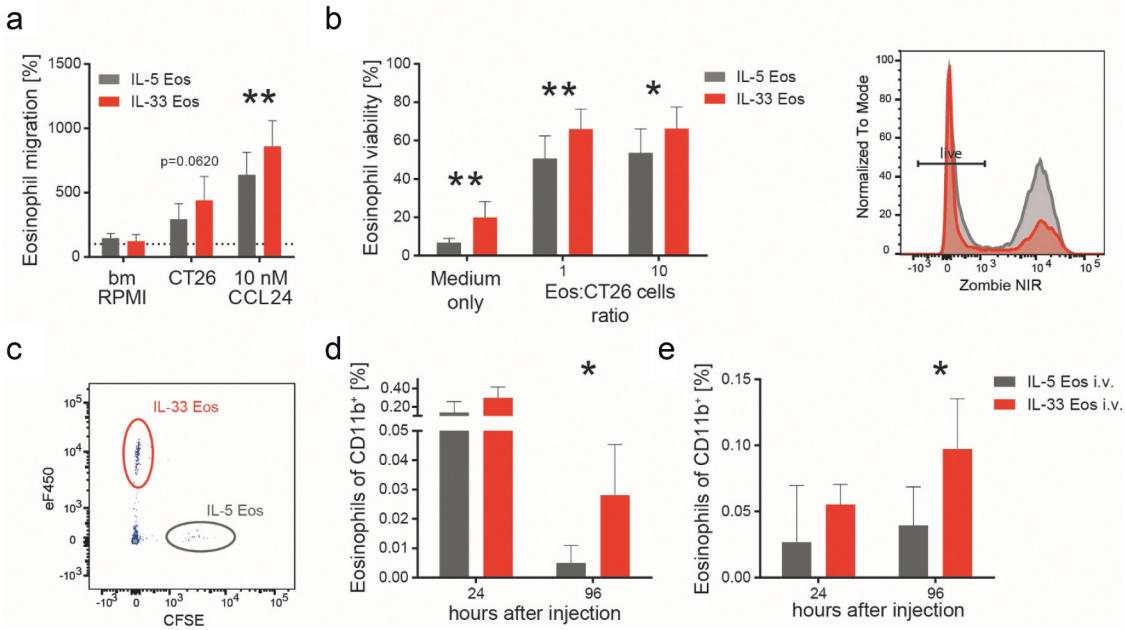

Figure 3. IL-33 dependent differences in migration and survival of eosinophils. (a) The migration of eosinophils (pre-activated with IL-33 [IL-33 Eos] or incubated with IL-5 alone [IL-5 Eos; serving as control]) toward CT26 cell-conditioned medium and toward CCL24 is shown. Data are means ± SD, pooled from three independent experiments (n = 7) and expressed as % of vehicle (bmRPMI). (b) Eosinophil viability was measured after 24 hrs co-incubation with indicated ratios of CT26 cells and medium only, and identified as Zombie NIR™ fixable viability dye (Zombie NIR) negative cells (means ± SD, n = 3). The histogram shows a representative experiment (grey = IL-5 Eos, red = IL-33 Eos). (c) A representative flow cytometry plot shows eosinophils (IL-33 Eos and IL-5 Eos) which were injected intravenously (i.v.) into ΔdblGATA-1 mice and detected in the blood after 24 hrs. IL-33 Eos (eFluor™ 450 [eF450]-stained) and IL-5 Eos (CFSE-stained) were identified as live/CD45$^+$/CD11b$^+$ cells. (D, E) Graphs show IL-33 Eos and IL-5 Eos (% of CD11b$^+$) in the blood (d) and in subcutaneous CT26 tumors 24 and 96 hrs after adoptive transfer of eosinophils into ΔdblGATA-1 mice (e); n = 4–5. Statistical differences were assessed by two-way ANOVA with Sidak's post hoc test, multiple *t*-tests and by unpaired student's *t*- test. *p < .05; **p < .01.

Figure 4i shows immunohistochemistry of s.c. tumors in ΔdblGATA-1 mice infiltrated with IL-33 Eos.

We not only applied IL-33 pre-incubated eosinophils into CT26 tumor-bearing ΔdblGATA-1 mice i.v., but also injected IL-33 i.p. into ΔdblGATA-1 mice repopulated with IL-5 Eos and we detected reduced tumor growth (*Supplementary* Fig. 5A-B), but, unlike in Figure 4h, this time the number of tumor-infiltrated eosinophils was not significaltly increased (i.p. IL-33 vs. i.p. vehicle [PBS], *Supplementary* Fig. 5 C). However, eosinophils in tumors of mice injected with IL-33 i.p. showed increased CD107a expression (vs. mice injected i.p. with PBS vehicle) indicating increased degranulation of eosinophils (*Supplementary* Fig. 5D). Collectively, the results demonstrate that IL-33 enhances the antitumor properties of eosinophils *in vivo*.

Eosinophils activated by IL-33 have increased cytotoxic potential

Due to the increased expression of degranulation markers in eosinophils after IL-33 treatment *in vitro* (Figure 2a) and in tumors of BALB/c and ΔdblGATA-1 mice (adoptively transferred with eosinophils) *in vivo* (Figure 2b and *Supplementary Fig.* 5), we investigated a possible mechanism by which eosinophils could contribute to tumor growth reduction that is associated with degranulation. We, therefore, looked at the eosinophil's potential of killing tumor cells. Eosinophil-mediated cancer cell killing was described earlier in different kinds of solid tumors, including CRC and melanoma.[7,25] We investigated this effect also in our *in vitro* settings and found that co-incubation of eosinophils with increasing ratios of CT26 cells for 6 hrs increased the number of dead CT26 tumor cells (Figure 5a). A stronger increase in dead CT26 tumor cells was observed after a 24 hr co-incubation (Figure 5a). Since it was shown that the activation of eosinophils with IL-33 could increase death in melanoma cells[25] we also tested this finding in our settings and co-incubated IL-33 Eos and IL-5 Eos with CT26 cells at different ratios. Compared to CT26 cells incubated with IL-5 Eos, the activation of eosinophils with IL-33 led to increased percentages of dead (Annexin-V$^+$) CT26 cells (Figure 5b,c).

Discussion

IL-33 is a cytokine (alarmin) released upon necrotic cell death, particularly, by wounded epithelial and endothelial cells.[14,44] In the context of cancer, it has been described as a pro- and anti-tumorigenic cytokine, depending on the tumor entity (reviewed in[16]). Recently, IL-33 has been linked with eosinophil activation, TATE and reduced tumor growth in a model of skin cancer.[13,25] By using two different CRC models, a s.c. tumor cell engraftment and an AOM+DSS-driven colitis-associated model, we can show that administration of IL-33 reduces tumor growth in CRC. The reduction is dependent on the presence and the activation status of eosinophils.

Several studies have previously addressed the role of the IL-33/ST2 axis in CRC showing either tumor promoting[17-24] or tumor reducing effects.[26,27,45,46] Concerning the tumor microenvironment, tumor promoting effects by IL-33 have been linked with Tregs, which suppress tumor immunity,[18,19] and

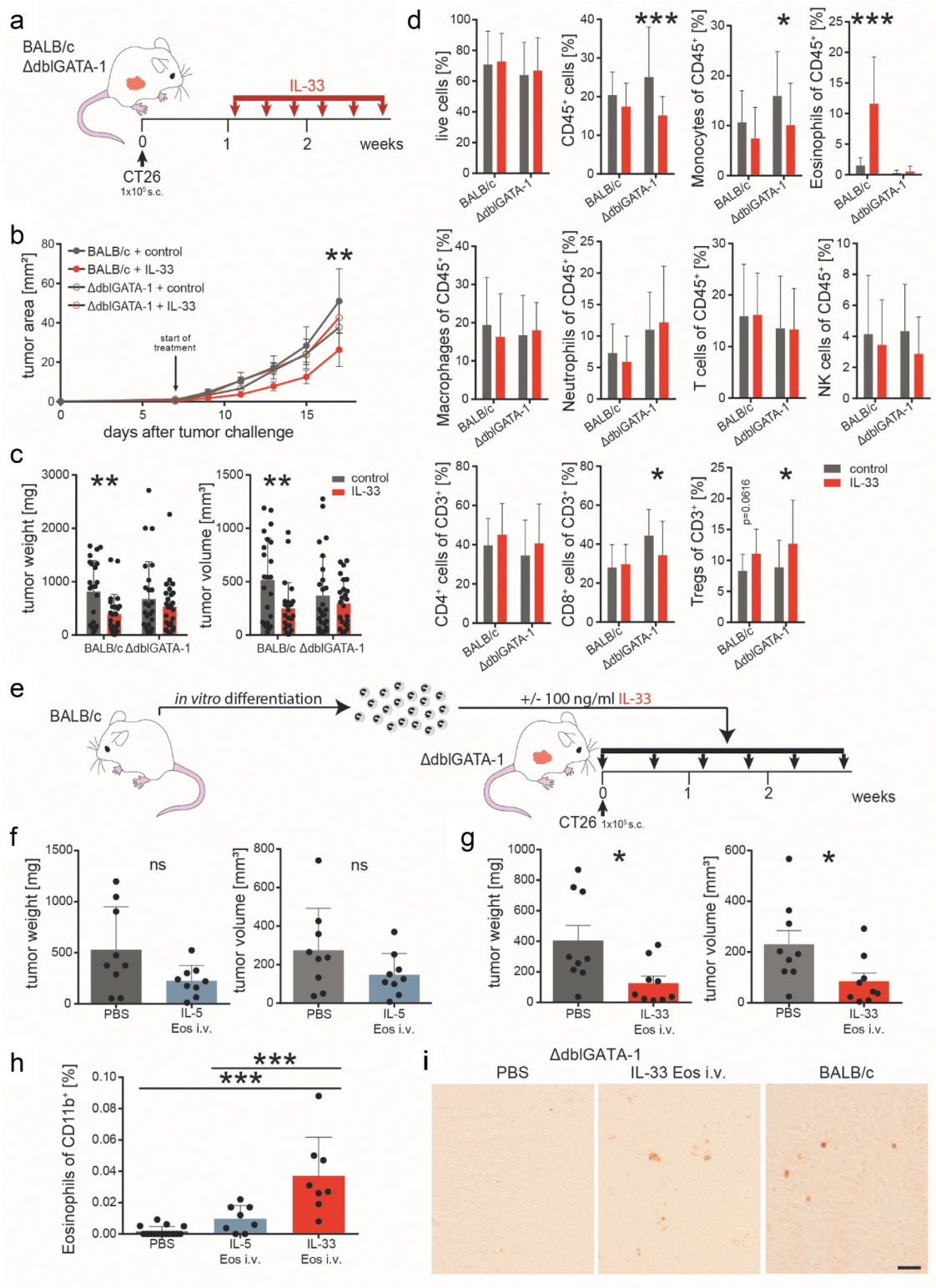

Figure 4. Eosinophils are necessary for a reduction in tumor growth by IL-33. (a) Schematic presentation of the subcutaneous (s.c.) tumor model performed in BALB/c and eosinophil deficient ΔdblGATA-1 mice. IL-33 was given i.p. at a concentration of 0.4 µg/mouse every other day for a total of six times. (b) Tumor growth was monitored during the course of the experiment; n ≥ 8. Data indicate mean values ± SEM. **p < .01 BALB/c + control vs. BALB/c + IL-33; no significance between other groups. (c) One day after the last IL-33 injection, BALB/c and ΔdblGATA-1 mice were sacrificed and tumor weight and volume were measured *ex vivo* showing no differences between IL-33- and vehicle (control) treatment in the ΔdblGATA-1 but tumor growth reduction in the BALB/c mice; n ≥ 23, three independent experiments. (d) Flow cytometric analysis of single cell suspensions of tumors from BALB/c and ΔdblGATA-1 mice indicating significant differences between IL-33- and vehicle-treated (control) ΔdblGATA-1 mice for $CD45^+$, monocytes, $CD8^+$ T cells and Tregs, as well for eosinophils between IL-33- and vehicle-treated (control) BALB/c mice; n = 24; three independent experiments. (e) ΔdblGATA-1 mice with s.c. tumors were repopulated by adoptive transfer (twice weekly) with either IL-33 Eos or IL-5 Eos (eosinophils were isolated from bone marrow of BALB/c mice and differentiated). (f) Adoptive transfer with IL-5 Eos failed to significantly restore tumor reduction in ΔdblGATA-1 mice (in comparison to ΔdblGATA-1 mice given PBS only). (g) However, adoptive transfer with IL-33 Eos (vs. PBS only) significantly restored reduction of tumor growth; n = 8–9. (h) Single cell suspensions of the s.c. tumors were prepared and infiltrated eosinophils were evaluated as % of $CD11b^+$ cells; n = 8–15. (i) Immunohistochemistry of s.c. tumors with an anti-EPX antibody shows that PBS-treated ΔdblGATA-1 mice are devoid of eosinophils. After adoptive transfer of IL-33 Eos (by i.v. injection), EPX staining is visible in the tumors of ΔdblGATA-1 mice. Infiltrated eosinophils in s.c. tumors of BALB/c mice are shown for comparison (representative images of n = 3/group; calibration bar: 20 µm). Statistical differences were assessed by using multiple *t*-tests, one-way ANOVA with Tukey's multiple comparisons test and unpaired student's *t*-test. *p < .05; **p < .01; ***p < .001, ns = not significant.

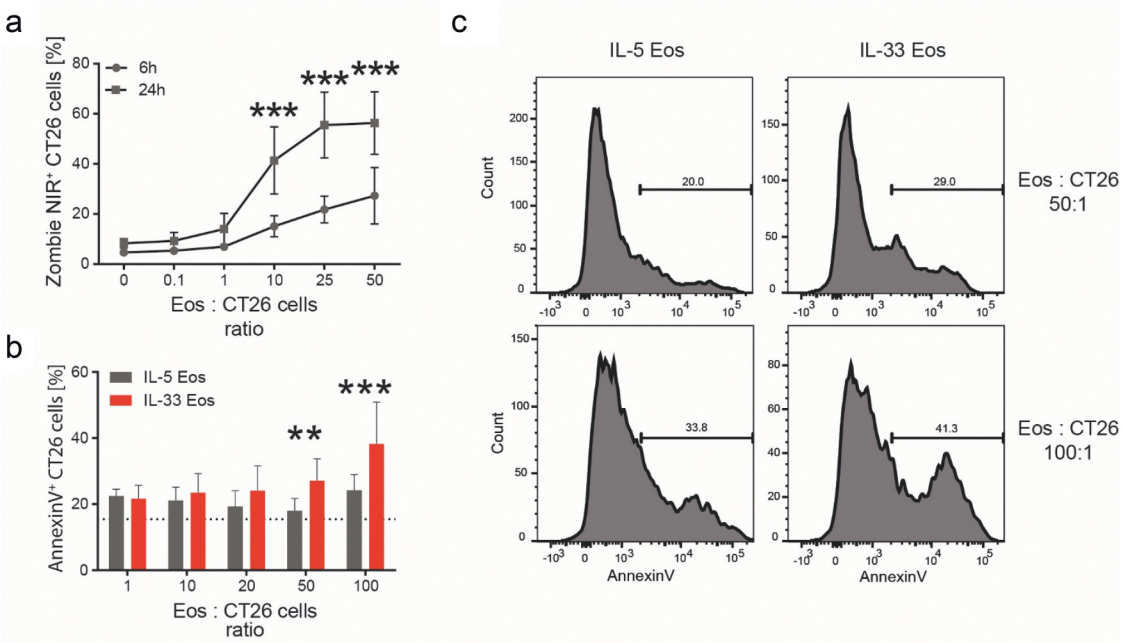

Figure 5. Cytotoxicity of eosinophils against CT26 cells *in vitro*. (a) Bone marrow-derived eosinophils (Eos) were co-incubated with CT26 cells at different eosinophil (Eos):CT26 cell ratios for 6 and 24 hrs. The percentage (%) of dead (Zombie NIR$^+$) CT26 cells is depicted as mean ± SD; n = 5. (b) CT26 cells were co-incubated with either IL-5 Eos or IL-33 Eos at different Eos:CT26 cells ratios for 7 hrs (n = 5). Percentages (%) of AnnexinV$^+$ CT26 cells are shown. (c) Representative histograms of IL-5 Eos and IL-33 Eos co-incubated with CT26 cells for 7 hrs at indicated ratios. Statistical differences were assessed using two-way ANOVA with Sidak's post hoc test. **p < .01, ***p < .001.

with macrophage infiltration into tumors to stimulate PGE_2 production that enhances colon cancer cell stemness.[20] In our present work, we primarily focused on the role of eosinophils in the microenvironment and whether they might play a role in IL-33-induced reduction in tumor growth. In accordance with a report in melanoma by Lucarini et al.,[25] we observed that IL-33 reduced the growth of tumors in mice engrafted with the colon cancer cell line CT26. The reduction was accompanied by an increased number of eosinophils and higher levels of IL-5 and CCL24 in the tumors (but not of CCL11 and CCL5) suggesting that these mediators likely contribute to increased eosinophil infiltration. Our observation that IL-33 can drive IL-5 and CCL24 expression is also supported by another study describing that in eosinophilia of airway inflammation, IL-33-stimulated production of IL-5 is accompanied by increased levels of CCL24, but not of CCL11.[47]

Despite several reports highlighting the influence of eosinophils on the nature of immune cell infiltrates in various solid tumor models,[12,25,48,49] we observed that IL-33-induced changes in the microenvironment of the CT26 tumors only occurred in CD4$^+$ T cells and Tregs but not M1 macrophages, myeloid derived suppressor cells (MDSCs), CD8$^+$ T or NK cells. In accordance with other studies,[18,19] Tregs slightly increased in CT26 tumors post-IL-33 treatment but this increase was not associated with a higher tumor burden. We also found a higher number of Tregs in tumors of ΔdblGATA-1 mice after IL-33 treatment vs. control with no change in tumor burden, suggesting that the increase in Tregs occurred independently of the presence of eosinophils. Unlike in the BALB/c mice, we also detected a decrease in CD8$^+$ T cells in the microenvironment of the ΔdblGATA-1 mice post IL-33 treatment. Given the fact that eosinophils produce chemokines for the attraction of CD8$^+$ T cells into the tumor microenvironment[12] the deficiency of eosinophils may have reduced the infiltration of CD8$^+$ T cells into tumors, however, it is not clear why this is only apparent after IL-33 treatment. Since IL-33 has been shown to promote tumor infiltration of CD8$^+$ T cells[16] one would have expected an increase in CD8$^+$ T cells after IL-33 treatment but we did not observe this effect in the BALB/c mice. As ΔdblGATA-1 mice have been also described to show disturbed immune formation in the intestines,[50] it is possible that less CD8$^+$ T cells were formed and that IL-33 could play some role in it. Furthermore, we detected reduced infiltration of monocytes in ΔdblGATA-1 mice post IL-33 treatment which has been previously shown in wild type mice with melanoma.[25] Considering the minor changes in the tumor microenvironment after IL-33 treatment, our data rather suggested a direct anti-tumorigenic effect of eosinophils on tumor cells. We, therefore, performed *in vitro* experiments by co-incubating eosinophils with CT26 cells and showed IL-33 dependent cytotoxic properties of eosinophils, corroborating data from studies in human and murine CRC and melanoma.[7,10,13,25]

That lymphocytes may not be needed for the effects of eosinophils on cancer growth has been previously reported in different models of heterotopically engrafted solid tumors,[51] as well as in colitis-associated CRC models and Apc$^{min/+}$ mice, which develop intestinal tumors spontaneously.[7] In our AOM +DSS-induced CRC model we detected IL-33-induced TATE, which was accompanied by reduced tumor growth, but we failed to see changes in the other leukocytes measured. The role of IL-33 was also investigated in colitis-associated CRC in

other reports, showing that genetic depletion of IL-33 leads to a disruption in the IgA-microbiota axis and to an increased number of tumors, but no data on the tumor microenvironment were included for the colitis-associated CRC model.[48] On the other hand, Eissmann et al. investigated genetic ablation of the IL-33 receptor ST2 in a sporadic mouse model of CRC applying AOM over several weeks.[26] In that study, genetic ST2 deficiency led to increased formation of colonic tumors, depending on the suppression of IFN-γ gene expression[26] (IFN-γ expression was unaltered in our study; unpublished data). Although ST2 deficiency did not affect the infiltration of $CD8^+$ T cells and myeloid cells into the tumors, it did increase the frequency of tumor-infiltrating Tregs.[26] At last, one study shows inhibiting effects of IL-33 on colon cancer cell growth,[45] however, we did not observe direct effects of IL-33 on CT26 cell proliferation (*Supplementary* Fig. 3). Collectively, these studies support our work on the role of IL-33 in reducing tumor growth in CRC.

IL-33 is known to lead to activation of eosinophils[52,53] which is accompanied by upregulation of molecules important for eosinophil transmigration (CD11b) and degranulation (CD63).[13,25,39,54] We could confirm these observations in our experiments for *in vitro*-activated eosinophils as well as for tumor-infiltrated eosinophils *in vivo*. We furthermore detected an increased expression of CD107a, which is, next to CD63, another marker of degranulation,[42] indicating that degranulation of eosinophils most likely represents a crucial mechanism of reducing growth of tumors in our experiments. The increased expression of Siglec-F on eosinophils in tumors of IL-33-treated mice is of particular interest because high Siglec-F expression has been associated with activation and migration of eosinophils to the airways.[40] This suggests that treatment with IL-33 may have made eosinophils more susceptible to migration and degranulation. Similarly, our migration assays with eosinophils *in vitro* demonstrated increased migration of IL-33 Eos toward CCL24 as compared to IL-5 Eos while migration toward CT26 cell-conditioned supernatant was less pronounced (p value .062).

Eosinophil depletion using anti-Siglec-F antibodies was used in the past to evaluate the engagement of eosinophils in tumor reduction.[12,25] A recently published article now suggests that eosinophil depletion by anti-Siglec-F antibodies does not result in reduction of eosinophil numbers but rather in an inhibited interaction of Siglec-F with its ligands leading to the observed biological effects.[55] Therefore, we used ΔdblGATA-1 mice in order to test whether the anti-tumorigenic effect of IL-33 treatment was dependent on the presence of eosinophils. IL-33 treatment of CT26 tumor-bearing ΔdblGATA-1 mice showed no decrease in tumor size. The repopulation of tumor-bearing ΔdblGATA-1 mice with eosinophils revealed that significantly smaller tumors developed only in mice that were injected with IL-33-activated eosinophils. In addition, more eosinophils infiltrated tumors in CT26 tumor-bearing ΔdblGATA-1 mice injected with IL-33 Eos vs. mice injected with IL-5 Eos. Our observations confirm data from a different study describing decreased growth of melanoma only when tumor cells were co-injected with IL-33-activated eosinophils.[13]

The viability of eosinophils may be significantly affected by tumor cells themselves. As recently demonstrated, the life span of eosinophils is extended independently of IL-5 when they are co-incubated with supernatant of colon cancer cells overnight.[7] In line with this finding, we could also detect an increase in viable eosinophils when they were co-incubated with CT26 cells. It is known that IL-33 prolongs eosinophil survival[52] and that CT26 cells produce IL-33.[27,56] Therefore, CT26 cell-derived IL-33 in our supernatants could have been a factor to increase survival of eosinophils. Finally, we also tested whether the effects of colon cancer cell supernatant and IL-33 were additive. Our findings showed that IL-33 enhances the extended survival of eosinophils in the CRC tumor microenvironment both *in vitro* and *in vivo*.

In conclusion, our study suggests that eosinophils play an indispensable role in the tumor growth-reducing effect of IL-33 in models of CRC. Reduction in tumor growth is significantly enhanced when eosinophils are activated by IL-33. Degranulation of eosinophils seems to be a major mechanism that contributes to the IL-33 dependent anti-tumorigenic effects while the change in the tumor microenvironment may play a minor role. Our data, therefore, strongly emphasizes the importance of eosinophils and their activation when considering IL-33 as a target of therapy against CRC.

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Author contributions

MK, CH, JK and RS designed and supervised the research. MK, PVC, KM, AS and CH did all the *in vivo* and *in vitro* experiments, collected data, and analyzed the results. MK did the statistical evaluation. ES, JK, AH and RS interpreted the data and provided technical support. MK and RS wrote the manuscript. All authors critically revised and commented on the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Review

The Immune Endocannabinoid System of the Tumor Microenvironment

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Abstract: Leukocytes are part of the tumor microenvironment (TME) and are critical determinants of tumor progression. Because of the immunoregulatory properties of cannabinoids, the endocannabinoid system (ECS) may have an important role in shaping the TME. Members of the ECS, an entity that consists of cannabinoid receptors, endocannabinoids and their synthesizing/degrading enzymes, have been associated with both tumor growth and rejection. Immune cells express cannabinoid receptors and produce endocannabinoids, thereby forming an "immune endocannabinoid system". Although in vitro effects of exogenous cannabinoids on immune cells are well described, the role of the ECS in the TME, and hence in tumor development and immunotherapy, is still elusive. This review/opinion discusses the possibility that the "immune endocannabinoid system" can fundamentally influence tumor progression. The widespread influence of cannabinoids on immune cell functions makes the members of the ECS an interesting target that could support immunotherapy.

Keywords: tumor microenvironment; endocannabinoid system; cannabinoid receptors; immune cells

1. Introduction

Gene mutations either caused by inheritance, environmental influence, faulty DNA replication or epigenetic modifications, and the accumulation and aberrant activity of these genes are key features in the process of cancer development [1,2]. Cells that aberrantly express these genes are constantly recognized and subsequently eradicated by cells of the immune system during tumorigenesis in a process called immune surveillance [3]. Nonetheless, mutated cells escape this process and succeed in developing cancer through the selection of tumor cell variants that either lack immunogenic features of recognition or exhibit features for the suppression of the evoked immune response [4].

Maintenance of tissue homeostasis is the work of immune cells, fibroblasts, the vasculature and extracellular matrix components. Apart from cancer cells, neoplastic lesions contain additional cell types, such as endothelial cells, pericytes, cancer-associated fibroblasts and immune cells [5]. Together, they can serve as a hurdle of cancer development [6]. Similar to inflammation, aberrant signaling, driven by cytokines and lipid mediators, among them also endocannabinoids, cause changes in tissue homeostasis and a shift towards a pro-tumorigenic environment and eventually to the development of cancer [6,7]. Thus, ongoing inflammation constitutes one of the hallmarks of cancer [5]. Like in inflammation, cells of the innate and adaptive immunity infiltrate tumors to form the immune tumor microenvironment (TME) with the aim to combat neoplastic growth [8]. Many of these cells express components of the endocannabinoid system (ECS), such as cannabinoid receptors [9–12]. Immune cells interact with each other and with tumor cells, they react to other components of the TME

and the ECS, and they can subsequently halt but also contribute to tumor progression in experimental and clinical cancer [8,13]. All types of immune cells can be observed in tumors, including macrophages, dendritic cells (DCs), neutrophils, eosinophils, mast cells, natural killer (NK) cells, and B and T cells (including Th cells, and cytotoxic T cells) [8]. Importantly, disease-free and overall survival critically depends on the immune cell compositions within the TME [8].

2. The Endocannabinoid System (ECS)

Many immune cells contain components of the ECS, an entity that regulates organ- and cell-specific physiological events with the aim to restore cell and tissue homeostasis. It includes the cannabinoid receptors 1 and 2 (CB_1 and CB_2), the endogenous ligands of the cannabinoid receptors, the so-called endocannabinoids, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), their enzymes for synthesis (diacylglycerol lipase (DAGL)), *N*-acylphosphatidyl-ethanolamine phospholipase D (NAPE-PLD) and degradation (fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL)), and their transporters [14,15]. The ECS is widely expressed throughout the body and can be found in almost all organs, however, the human nervous system and the immune system have been found to represent the highest expression levels of cannabinoid receptors [16]. Parts of a wider ECS network are (i) non-cannabinoid receptors that show responsiveness to cannabinoids, such as G protein-coupled receptors 55 and 18 (GPR55, GPR18), PPAR receptors, TRP- and 5HT3receptors, potassium channels, and (ii) endocannabinoid-like lipids such as oleoyl- and palmitoyl-ethanolamide (OEA and PEA). These components belong to an expanded ECS (endocannabinoidome; [17]). See Pertwee [18] and Cristino et al. [15] for a more detailed description of the ECS and the "endocannabinoidome".

3. The Endocannabinoid System and the Tumor Microenvironment

This review discusses the potential role of (endo)cannabinoids and other ECS components in immune cells that are typically found in the TME. For detailed effects of cannabis/(endo)cannabinoids on tumor cells and cannabinoid receptor signaling, the reader is referred to several other recent reviews [19–22].

Receptors and enzymes of the ECS have been mostly measured and quantified by immunohistochemical, Western blot and PCR methods using tissue from a variety of tumor models and biopsies from patients with, e.g., breast, brain, prostate, colon and cervical cancer. Each of the tumors may exhibit either up- or down-regulation of cannabinoid receptors (which are often increased in tumors), and of endocannabinoids and their metabolizing enzymes, FAAH and MGL (rev. in [20]). Correlations between expression of cannabinoid receptors and disease outcome largely differ between various types of cancer [20] indicating that there is no universal (e.g., anti-carcinogenic) role of the ECS in tumor development but that its role rather depends on the type of the tumor. For instance, CB_2 overexpression in HER-2 positive breast cancer is a marker for poor outcome [23], whereas in hepatocarcinoma, CB_1 and CB_2 expression correlate with good clinical outcome [24].

(Endo)cannabinoids have direct anti-carcinogenic effects on tumor cells [19,25,26]. These effects include inhibition of proliferation, cell cycle arrest, apoptosis and autophagy [26,27]. Thus, AEA- and 2-AG-dependent anti-proliferative effects have been demonstrated in colon, breast, prostate and cervical cancer cells [20,28,29]. Many of these studies were also conducted with exogenous cannabinoids such as Δ^9-THC, which mimics the effects of endocannabinoids on cannabinoid receptors [19]. In this context, however, Δ^9-THC has shown biphasic effects, inducing cancer cell growth at low (100–300 nM) [30] and cell death at high (μM) concentrations [31].

While there is ample evidence that cannabinoids and components of the ECS are involved in inhibiting tumor cell proliferation in vitro, little is known about the impacts the ECS has on cells of the TME and consequently on tumor progression. A study by Busch et al. demonstrated that in models of lung adenocarcinoma with different types of mutation (in Kras, p53, or Egfr), the immune cell content varied, suggesting that immune responses and TME landscape of tumors critically depend on tumor cell mutations [32]. As for the ECS, its components are located in immune cells (see Figure 1)

besides their expression in tumor cells. Among the few studies that have addressed the ECS in the TME, our group showed, by use of a chemically induced colorectal cancer model, a marked shift in the composition of the immune TME in GPR55 knockout vs. wildtype mice. Knockouts displayed a lower amount of MDSCs which suppress anti-tumor immunity [33], but a higher number of $CD4^+$ and $CD8^+$ cells (which correlate with better prognosis) [34]. Among the other studies, Qiu et al. reported that 2-AG induced the expansion of MDSCs in a model of pancreatic adenocarcinoma with no effect on $CD4^+$ and $CD8^+$ cells [29]. In a model of colon cancer with mice bearing MGL-deficient macrophages, a lower tumor burden was observed in knockouts as compared to wildtypes in a study by Xiang et al. (2018) [35]. Zhu et al. demonstrated that Δ^9-THC suppressed host immune reactivity to lung cancer via inhibitory cytokines [36].

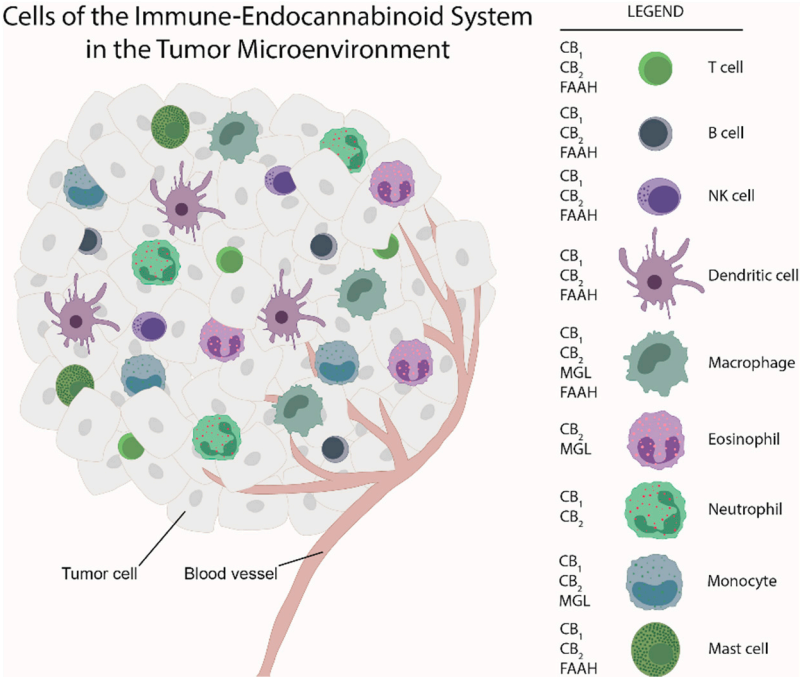

Figure 1. Tumors locally recruit immune cells that reject and also promote tumor development and metastasis. Immune cells express components of the endocannabinoid system, thereby they are able to form an "immune endocannabinoid system" within the tumor microenvironment. Most of the immune cells express cannabinoid receptors (CB_1, CB_2) and enzymes for endocannabinoid degradation (monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH)). In addition, they are known to release endocannabinoids (see Table 2).

To date, these data suggest that exogenous cannabinoids and ECS components have an influence on immune cells of the TME and that the ECS could be involved in the control of this immune cell network and hence in tumor growth.

Before we discuss ECS components of immune cells and their relation to the TME, a brief introduction of the role of immune cells (expressing ECS components) in the TME is given in the following sections.

4. Immune Cells in the Tumor Microenvironment

4.1. T Lymphocytes

4.1.1. $CD8^+$ T Cells

Tumors are infiltrated by various T cell populations that preferentially reside in the invasive tumor margin and the draining lymphoid organs [8]. Among these populations, $CD8^+$ T cells are capable of

detecting tumor cells via recognition of aberrant antigens from overexpressed or mutated molecules that are presented by major histocompatibility complex I (MHC I) [37]. After antigen and MHC I recognition, cytotoxic molecules, such as granzymes and perforin, are released by $CD8^+$ T cells and result in tumor cell killing [38]. Other mechanisms that underlie the killing of tumor cells via $CD8^+$ T cells include the death receptors TRAIL and FasL (reviewed by Martínez-Losato and colleagues [39]). Increased numbers of $CD8^+$ T cells in tumors are associated with a better clinical outcome in patients, e.g., with breast [40,41] and colorectal cancer [42,43], and glioblastoma [44]. However, anti-tumorigenic lymphocytes may become exhausted or dysfunctional due to the engagement of effector molecules or inhibitory receptors (e.g., T cell immunoglobulin domain and mucin domain protein 3 (TIM-3), cytotoxic T lymphocyte antigen-4 (CTLA-4) and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT)) of tumor and TME cells [45,46]. Furthermore, engagement of programmed cell death protein ligand-1 (PD-L1) on tumor cells with programmed cell death protein-1 (PD-1) expressed on $CD8^+$ T cells reduces the susceptibility of tumor cells to T cell-mediated killing, inducing tumorigenesis [47]. In recent years, antibodies targeting those inhibitory molecules and their ligands were moving into the focus of immunotherapies, namely as immune checkpoint inhibitors (ICI), with CTLA-4 and PD-1 being the most successful targets (reviewed in [46]).

4.1.2. $CD4^+$ T Cells

The infiltration of different $CD4^+$ T cell subpopulations has been described in solid tumors [48,49]. $CD4^+$ T helper 1 (Th1) cells mediate anti-tumor effects with the help of $CD8^+$ T cells [50], hence, elevated numbers of $CD4^+$ T helper 1 cells in the TME correlate with a positive clinical outcome in various human tumors (rev. in [8]).

For other T helper cell populations, e.g., Th2, Th17 or Th22, a role in the TME and in tumor growth has been suggested, however, the effects on tumor development are contradictory [8,51–54].

Other immunosuppressive $CD4^+$ T cells, i.e., regulatory T cells (Tregs), are often described as pro-tumorigenic (reviewed in [8]). Elevated numbers of Tregs inversely correlate with the survival of patients with ovarian [55] and breast cancer [56], and hepatocellular carcinoma [57], although the opposite was observed for follicular [58] and Hodgkin's lymphoma [59]. Mechanisms linked to the suppressive function of Tregs include secretion of suppressive cytokines, cytolysis of effector T cells, metabolic disruption, and DC suppression (reviewed in [60]).

4.2. B Lymphocytes

B cells are important cells of the humoral immunity and infiltrate tumors where they mostly localize to tertiary lymphoid structures (TLS) [61]. B cell-mediated tumor cell killing can be directly accomplished via the Fas/FasL or TRAIL/Apo2L pathways [62,63], or indirectly via production of IFN-γ [64], thus recruiting and activating NK cells and polarizing T cells towards Th1 [61]. Another anti-tumorigenic effect of B cells is mediated by anti-tumor antibodies, as recently reviewed by Sharonov et al. [61]. Cancer-specific neo-antigens, such as mutated p53 [65], but also self-proteins represent targets for antibodies in tumors [66,67]. Infiltrating B cells were reported as an important predictor for good clinical outcome in patients with metastatic melanoma [68]. Further studies identified a correlation of peritumoral B cells [69] and infiltrating B cells [70,71] with reduced relapse rates and prolonged survival in cervical and lung cancer, respectively.

However, B cells and antibody production may not be only associated with less tumor growth. As to the prognostic role of anti-p53 antibodies, contradictory results were reported in cancer patients [72]. Antibody production in melanoma-draining lymph nodes accelerated tumor growth, although this was attenuated by macrophages [73]. Not only antibodies may promote tumor growth, but also an immunosuppressive subpopulation of B cells (regulatory B cells) [61]. This population assists the generation of Tregs [74,75] and expresses inhibitory ligands [76] and cytokines (i.e., IL-10 [77]), resulting in inhibition of $CD4^+$ and $CD8^+$ T cells and the promotion of tumor growth [78]. It is, therefore,

not surprising that B cells have been reported to be a sign for negative overall survival in various human cancer types such as, bladder, breast, and colorectal cancer, amongst others (reviewed in [61]).

4.3. NK Cells

NK cells are part of the innate immune system and eliminate cells with aberrant or absent expression of MHC I [79], a mechanism used by cancer cells to escape recognition by $CD8^+$ T cells [80]. Killing of tumor cells by NK cells is mediated by the release of lytic granules that contain perforin and granzymes, resulting in tumor cell apoptosis [81]. Death receptor-mediated killing of tumor cells via TRAIL and FasL is also harnessed for cancer cell elimination [82]. Generally, NK cells predict a good prognosis for many solid tumors [83], while sparse NK cell function was reported to correlate with the development of metastases in pharyngeal [84], head and neck [85] and other solid tumors [86]. An anergic/exhausted phenotype of NK cells has been reported in the stroma of lung [87] and colon cancer [88]. NK cell may well depict a target for cancer immunotherapy strategies, as recently reviewed [89].

4.4. Neutrophils

Until recently, neutrophils were only regarded as bystanders in cancer [90], however, they constituted 20% of all $CD45^+$ cells in non–small-cell lung cancer (NSCLC) tumor specimens, thus representing a major immune cell type in NSCLC [91]. They also represent a considerable portion of the infiltrating immune cells in other cancer types [92]. In NSCLC, neutrophil infiltration results in depletion of $CD4^+$ and $CD8^+$ T cells [93]. Clinical studies established a pro-tumorigenic role of tumor-associated neutrophils (TANs), for instance, in renal cell carcinoma [94], colorectal cancer [95], and cervical cancer [96]. In these studies, infiltrated neutrophils were associated with poor survival, thus, they were suggested to support the development of a malignant phenotype. In contrast, elevated numbers of TANs in stage II colorectal cancer resulted in improved survival [97]. TANs were also reported to be a favorable prognostic factor in gastric and colorectal cancer [98,99]. The role of neutrophils, therefore, in the TME seems contradictory, suggesting a certain plasticity between a pro- (N2-neutrophils) and anti-tumorigenic (N1-neutrophils) state [100]. Thus, Zhang and Houghton proposed TANs as targets for immunotherapy [101], and to either drain TANs completely or to focus on the pro-tumorigenic molecules secreted by TANs. In fact, the use of SX-682, a CXCR1/2 inhibitor, proved to be beneficial in a combined treatment regimen with ICI therapy in a mouse model, suggesting enhanced efficacy of ICI by neutrophil antagonism in NSCLC patients [93].

4.5. Eosinophils

Eosinophils are important immune regulators [102]. As such, it is likely that eosinophils can fundamentally shape the TME (rev. in [103,104]). Since they show pro- as well as anti-tumorigenic effects, Varricchi et al. suggested that different tumor entities accompanied with differences of the surrounding milieu affect the function of tumor-associated eosinophils [103]. Eosinophilia of blood and tumors are favorable for the outcome of several types of cancer (rev. in [103]). In experimental tumors, activated eosinophils either reduce tumor growth directly via degranulation [105,106] or, additionally, via recruitment of other anti-tumorigenic leukocytes [107,108]. In contrast to their anti-tumorigenic features, an association with tumor growth was also reported (rev. in [103]). Given that eosinophils are regular cells of the TME, they should be considered as important players that may likely influence immunotherapy of cancer patients [104].

4.6. Mast Cells

Mast cells belong to the innate immune system and contribute to various diseases, including cancer [109]. Infiltration of mast cells has been suggested to be either anti- or pro-tumorigenic in clinical cancer (rev. in [7]). In experimental tumor models, the role of mast cells is also contradictory [7]. Interestingly, tumor progression was unaffected by the presence of mast cells in colorectal [110],

and renal cancer [111]. In summary, studies suggest a cancer-specific role for mast cells and their effector molecules, but still many questions need to be answered before mast cells can be considered as a therapeutic target [7].

4.7. Monocytes

Monocytes are cells of the innate immune system, circulating in the blood before trafficking into the tissue. They maintain tissue homeostasis, support immunity, and suppress excessive immune responses [112]. Under pathological conditions, recruitment of monocytes to the site of inflammation and tumors is enhanced [113]. Recent studies described conflicting roles of monocyte subsets in cancer: on the one hand, inflammatory monocytes promote tumor growth [114] and correlate with poor clinical outcome [115], but, on the other hand, patrolling monocytes can hamper the development of metastases [116]. Monocytes trafficking into tumors differentiate either into DCs, especially into monocyte-derived DCs (moDCs) or into tumor-associated macrophages (TAMs) [113,117]. They can also maintain their state and be part of a monocyte pool [118].

4.8. Tumor-Associated Macrophages (TAMs)

TAMs were identified in the stroma of various types of cancer [117]. Elevated numbers of TAMs were reported to be linked with poor prognosis in human cancers, such as gastric, breast, bladder, and ovarian cancer, and Hodgkin's lymphoma [119,120]. Experimental studies, which support these clinical findings, revealed mediators secreted by TAMs as driving factors of tumor progression, either directly or indirectly [117]. Thus, VEGF-A produced by TAMs was shown to regulate angiogenesis and tumor progression in the PyMT (polyoma middle T oncoprotein) mouse model of breast cancer [121]. Other mechanisms include the rearrangement of the tumor architecture (e.g., via MMPs [122]), the induction of tumor cell proliferation and survival (e.g., through epidermal growth factor [123] or IL-6 [124]), and the suppression of anti-cancer immunity (e.g., via PD-L1 [125]). On the other hand, elevated presence of TAMs in colon [119,126], gastric [127] and nests of endometrial cancer [128] was linked to improved survival in clinical studies.

The influence of TAMs on tumor development may be better understood via the identification of their functional state [117]. Thus, macrophages are broadly sub-classified into M1 (classically activated) and M2 (alternatively activated) cells, differing in the cytokines needed for their polarization and effector functions [129]. M1-polarized macrophages have been reported to exert anti-tumorigenic functions. On the contrary, M2 macrophages are associated with immunosuppression [130], as well as with increased secretion of molecules for tissue remodeling, repair and angiogenesis [121,131]. Given the different functions of macrophage subtypes in tumor development, macrophages depict an attractive target for immunotherapies, either via depletion or via reprogramming towards anti-tumorigenic activities (reviewed in [132]).

4.9. Dendritic Cells (DCs)

DCs are antigen-presenting cells which screen their environment for antigens, followed by activation of cells of the adaptive immunity [133]. Increased infiltration of classic DCs (cDCs) into the TME is associated with a better disease outcome in lung cancer and melanoma [134–136]. Accumulation of plasmacytoid DCs (pDCs), another DC subset, has been reported in cancer, however, infiltration of this subset was associated with poor survival in breast cancer [137] and melanoma [94].

4.10. Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are of neutrophilic (PMN-MDSC) or monocytic origin (M-MDSC), and they are mainly detected in patients (or animals) with ongoing inflammation, infection or cancer [138,139]. MDSCs exert their suppressive function via various pathways. For example, they have been reported to induce the development of Tregs and the suppression of T cells [140,141]. M-MDSCs may also differentiate into TAMs and inflammatory DCs in the TME [142]. Furthermore, they may promote tumor development by

enhancing cancer stemness [143] and angiogenesis [144]. It is, therefore, not surprising that increased frequencies of circulating MDSCs have been associated with poor disease prognosis in breast cancer melanoma patients [145].

5. Cells of the "Immune Endocannabinoid System"

Many, if not all, immune cells express cannabinoid receptors and are able to produce and degrade endocannabinoids (rev. in [146]; Figure 1; actions of (endo)cannabinoids and cannabinoid receptor ligands on these immune cells are summarized in Table 1).

Therefore, the ECS members virtually constitute an "immune endocannabinoid" system ([147]; see Table 2 for ECS member expression in immune cells). In a study by Galiègue et al., human leukocytes were shown to express CB_2 receptors at the following levels: B cells > NK cells > PMN >neutrophils > $CD8^+$ T cells > monocytes > $CD4^+$ T cells, however, significantly less (or no) expression of CB_1 was observed in these cells [16], indicating a regulatory role in immune cell function primarily for CB_2 [148]. It should be noted though that expression levels of cannabinoid receptors may vary in these cells depending on the cells' localization and environment. The presence of inflammatory cytokines (IL-6, IL-1β and TNF-α), for instance, was shown to increase CB_1 and CB_2 expression in peripheral blood mononuclear cells [149]. Since these cytokines are present in tumors and often increased (promoting growth; [150]), cannabinoid receptor expression in immune cells may be significantly higher in tumors than in healthy tissues. In line, tumors of the brain, prostate, pancreas and cervix all show higher levels of CB_1 and CB_2 receptors as compared to normal tissue [20].

Table 1. Some main actions of (endo)cannabinoids and cannabinoid receptor ligands on immune cells.

Immune Cells	Effects of (Endo)Cannabinoids or Synthetic Cannabinoid Receptor Ligands	(Endo)Cannabinoids/Ligands	Reference
T cells (human, mouse)	Inhibition/induction of Th1 and Th2 cytokines	Δ^9-THC	[151–154]
T cells (human)	Suppression of proliferation and cytokine release via CB_2	AEA	
	Induction of apoptosis	AEA	
	Inhibition of migration	AEA	
B cells (human)	Stimulation of migration	2-AG	[153,155–157]
	Inhibition of proliferation	AEA	
B cells (mouse)	Stimulation of migration	2-AG	
NK cells (human)	Stimulation of migration via CB_2	2-AG	[158]
Dendritic cells (human)	Inhibition of cytokine production in myeloid and plasmacytoid dendritic cells	AEA	[159,160]
Dendritic cells (mouse)	Inhibition of Th1 and Th17 lineage induction	AEA	
	Stimulation of migration	2-AG	
Macrophages (mouse)	Stimulation of ROS production via CB_1	AEA, ACEA	[161–165]
	Inhibition of TNF-α production	2-AG	
	Suppression of ROS	2-AG	
Macrophages (human)	Inhibition of migration via CB_2	Δ^9-THC	
	Rapid actin polymerization via CB_2	2-AG	
	Stimulation of migration	2-AG	
Eosinophils	Stimulation of migration via CB_2	JWH133, 2-AG	[166,167]
Neutrophils (human)	Activation (MPO release, Ca^{++} mobilization)	2-AG	[168–170]
	Suppression of migration	JWH015, 2-AG	
	No effect on migration	Δ^9-THC	
Mast cells (human)	Control of degranulation via CB_1	AEA, ACEA	[171]

2-AG, 2-arachidonylglycerol; AEA, anandamide; ACEA, arachidonyl-2'-chloroethylamide (CB_1 agonist); CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; Δ^9-THC, delta 9-tetrahydrocannabinol; MPO, myeloperoxidase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha; JWH015 and JWH133 are CB_2 agonists.

Table 2. The immune endocannabinoid system.

	CB_1 Receptors (Species; Method of Detection)	CB_2 Receptors (Species; Method of Detection)	MGL (Species; Method of Detection)	FAAH (Species; Method of Detection)	Production of Endocannabinoids
PBMC	-human; PCR, FC, WB; T cells activated with TNFalpha [149] -human; PCR; [172] -human; PCR; [161]	-human; PCR, FC, WB; T cells activated with TNF-α; [149] -human; PCR; CB_2 3 x higher than CB_1; [172] -human; PCR; [161]			
Lymphocytes	-human; PCR, WB [173]			-human; ELISA, PCR [173]	AEA [173] 2-AG [174]
T cells	-human; PCR; T cells activated with CD3/28; [175] -human; PCR; T cells activated with TNF-α; [149] -human; PCR; [176]	-human; PCR; T cells activated with CD3/28; [175] -human; PCR [16] -human; PCR; T cells activated with TNFalpha [149] -human; FC; [177] -human; PCR; [176]		-human; PCR; [176]	
B cells	-human; PCR; [176]	-human; PCR; [16] -human; FC; [177] -human; FC; [178] -human; PCR; [176] -human, FC; [179]		-human; PCR; [176]	
Monocytes	-human THP monocytes; PCR; [161]	-human; PCR; [16] -human THP monocytes; PCR; [161] -human, FC; [179]	-human; WB; [180]		2-AG [174]
Macrophages	-human PMA-treated monocyte-derived macrophages; PCR; [161] -mouse RAW264.7 cells; PCR; [161] -human PBMC-derived macrophages; PCR; [181] -rat; circulating macrophages; PCR; [182]	-human; PCR; [16] -human PMA-treated monocyte-derived macrophages; PCR; [161] -mouse RAW264.7 cells; PCR; [161] -human; differentiated monocytes; PCR; [183] -mouse tumor-associated macrophages; PCR; [35]	-mouse; tumor associated macrophages; [35]	-rat; circulating macrophages; PCR; [182]	AEA in RBL-2H3 basophils, J774 and RAW264.7 mouse macrophages [182,184–186] 2-AG in mouse peritoneal macrophages [187] 2-AG in J774 cells [182] 2-AG in mouse P388D1 macrophages [188] 2-AG in mouse peritoneal macrophages [189] 2-AG in RAW264.7 cells [186]
NK cells	-human; PCR; [176]	-human; PCR; [16] -human; FC; [177] -human; PCR; [176] -human, FC; [179]		-human; PCR; [176]	
Dendritic cells	-human; PCR, WB; [190]	-human; PCR, WB; [190]		-human; PCR, WB; [190]	AEA, 2-AG [190]

Table 2. *Cont.*

	CB_1 Receptors (Species; Method of Detection)	CB_2 Receptors (Species; Method of Detection)	MGL (Species; Method of Detection)	FAAH (Species; Method of Detection)	Production of Endocannabinoids
Neutrophils	-mouse bone marrow neutrophils (liver injury model); PCR; IF; [191]	-human; PCR; [16] -human; FC; [169] -not detected [168] -mouse bone marrow neutrophils (liver injury model); PCR; IF; [191] -human; WB; [192] -human, FC; [179]			2-AG [174]
Eosinophils		-human; PCR; [168] -human; PCR, Northern Blot; [167] -human; FC, [166]	-human; PCR; [168]		2-AG [174]
Mast cells	-rat RBL2H3 cells; PCR; [193] -mouse (primary BMMCs); WB; [194] -human mucosal-type mast cells; IHC; [171]	-rat RBL2H3 cells; PCR; [193] -mouse (primary BMMCs); WB; [194]		-human mast cells (HMC-1); FAAH activity measured; [195]	

AEA, anandamide; 2-AG, 2 arachidonoylglycerol; BMMC, bone marrow-derived mast cell; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; ELISA, enzyme-linked immunosorbent assay; FAAH, fatty acid amide. hydrolase; FC, flow cytometry; IF, immunofluorescence; IHC, immunohistochemistry; MGL, monoacylglycerol lipase; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PMA, phorbol 12-myristate. 13-acetate; WB, Western blot.

CB_1 and CB_2 Receptors in Immune Cells

As it can be seen from Table 2, most information on ECS components in immune cells is available for CB_1 and CB_2 receptors. They are expressed in almost all types of immune cells, suggesting that the inflammatory behavior of these cells is regulated through endocannabinoid activation. Both receptors couple to Gi/o proteins and inhibit adenylyl cyclase, but show some differences in other downstream effects. For instance, CB_2 receptors do not couple to potassium channels, such as the G-protein-gated inwardly rectifying potassium (GIRK) channels, providing an explanation as to its functional difference to CB_1 (rev. in [196]). In inflammatory conditions, CB_1 as well as CB_2 receptors may show equal actions, e.g., reduction of VEGF-A secretion in LPS-stimulated neutrophils [197] and human lung-resident macrophages [198]. However, CB_1 and CB_2 activation may also have opposing effects, especially with regard to reactive oxygen species (ROS) production and polarization of macrophages. In macrophages, activation of CB_1 enhances production of ROS and TNF-α, while activation of CB_2 suppresses these effects [161]. Additionally, CB_1 induces polarization towards an M1 phenotype [199], whereas activation of CB_2 causes a shift towards an anti-inflammatory M2 phenotype [200].

The role of CB_2 in inflammation and immune cell function is well described, but unanswered questions remain as to whether activation of CB_2 is anti- or pro-inflammatory [196]. For instance, activation of CB_2 by AEA reduces proliferation and inhibits release of pro-inflammatory cytokines, such as IL-2, TNF-α and IFN-γ, from primary T-lymphocytes; furthermore, IL-17 secretion from Th17 cells is suppressed [152]. In addition, CB_2 decreases expression of inflammatory cytokines [201] and accumulation of oxLDL in macrophages [202], indicating anti-inflammatory actions of CB_2. On the other hand, migration of B cells, dendritic cells and eosinophils [156,160,166,167], and adhesion of monocytes and macrophages is increased by 2-AG [164,165,203], also suggesting pro-inflammatory actions of CB_2.

In vivo effects of cannabinoid receptors have been mostly associated with anti-inflammation. However, pro-inflammatory in vivo effects of endocannabinoids have been also described, suggesting that actions of endocannabinoids (and especially of their metabolites) on cannabinoid receptors, and also the role of non-CB_1/CB_2 cannabinoid-related G protein-coupled receptors in inflammation are not yet fully understood (rev. in [146,204]).

6. The "Immune Endocannabinoid System" in Adaptive and Innate Immunity

From in vitro/vivo experiments with exogenous cannabinoids, there is solid evidence that cannabinoids influence key functions of immune cells, such as proliferation, migration, antibody formation, cytolytic activity, differentiation and apoptosis (rev in [146,147,151,205–207]. All of these functions are relevant for the immune cell composition of the TME and tumor growth.

Among the adaptive immune system, T cells are particularly influenced by cannabinoids, repeatedly shown by in vitro experiments using Δ^9-THC as an agent [147]. Thus, strong influence on proliferation of T cells by cannabinoids has been demonstrated in vitro [153] as well as in vivo [147]. In particular, regulation of $CD8^+$ T cell function, which, owing to their tumoricidal activity, critically determine cancer growth, is of importance. Together with $CD4^+$ Th1 cells, they are associated with good disease prognosis [208,209]. Differentiation of T cells may represent another function influenced by the ECS in the TME. A previous study showed that perinatal exposure to Δ^9-THC caused a marked alteration in T cell subpopulations, which was dependent on CB_1 and CB_2 [210]. In line with the cannabinoids' effects on T cell function and development, Yuan et al. demonstrated that Δ^9-THC regulates Th1/Th2 cytokine balance in activated human T cells [211]. B cells are another immune cell population whose differentiation depends on ECS components such as CB_2 receptors [177,178].

Therefore, $CD8^+$, $CD4^+$, and B cells could be highly susceptible to the effects of endocannabinoids within the TME, owing to their expression of CB_2 and CB_1 receptors (see Table 2).

Marijuana is well known for its immunosuppressive effects [212]. For instance, Δ^9-THC has been shown to worsen Legionella, herpes simplex and Listeria infections (rev. in [146,213] and to lower the number of T cells in mice after daily treatment (s.c.,10 mg/kg for 14 days) [214]. In addition,

in humans, cell-mediated immunity and host defense is suppressed by Δ^9-THC [215]. A study in cannabis users revealed a reduction in lymphocyte functionality and NK cell number, and a disruption of the Th1/Th2 balance [216], which could be associated with increased infection and impairment of cytokine production. Smoking of cannabis is also connected with alterations in the basal levels of CB_1 and CB_2 from PBMCs [172].

In line with their immunosuppressive role, cannabinoid ligands have been demonstrated to suppress phagocytosis, cell spreading, antigen presentation and other features of immune cells [217–220], all of which are essential for immune cell regulation in the TME. CB_2 receptor agonists, in particular, cause immunosuppression (rev. in [221]) as highlighted by a study from Zhu et al. who showed that activation of cannabinoid receptors by Δ^9-THC inhibited anti-tumor immunity through an CB_2-mediated increase in tumor promoting cytokines [36]. However, CB_2 may have multiple roles in the TME since the receptor can stimulate migration of myeloid leukemia cells and normal splenocytes [156]. Furthermore, CB_2 activates macrophages [35] and induces apoptosis in immune cells [222].

Certain innate immune cells of the TME, including neutrophils, M2 macrophages and MDSCs, have been associated with tumor progression [208]. Since ECS components are expressed in innate immune cells (see Table 1), endocannabinoid signaling in these cells may influence functions relevant for tumor growth. Macrophages are highly responsive to cannabinoids in terms of cytokine secretion, migration, phagocytosis and antigen presentation (rev. in [151]). They express cannabinoid receptors [35,181,183] and MGL [35,223], and a CB_2 dependent pro-tumorigenic role of macrophage-expressed MGL has been recently described [35]. In addition, Δ^9-THC was shown to inhibit Th cell activation through macrophages derived from CB_2 wildtype, but not from CB_2 knockout mice [224], indicating that macrophages and CB_2 are important in directing T cell responses.

Neutrophils exert immunosuppressive properties [225] and are the most prevalent immune cell type in non–small-cell lung cancer [91]. The gene signature of neutrophils predicted mortality better than any other immune cell signature in a cohort of >18k patients [226]. Neutrophils express CB_2 receptors [16]; the receptors suppress migration [169] and inhibit cell differentiation when overexpressed in myeloid precursor cells [227]. Hedge et al. recently showed that activation of cannabinoid receptors with Δ^9-THC mobilized MDSCs (which contain various forms of PMNs) and led to immunosuppression [228]. It was previously demonstrated that CB_2 (cooperating with GPR55) is involved in human neutrophil function [192]. The precise role of the ECS in tumor-associated neutrophils, however, remains elusive.

Dendritic cells (DCs) are also integral part of the TME and are essential in staging an adaptive immune response [208]. These cells have now moved into the light of anti-tumor therapy as their presence may promote susceptibility to immunotherapy [229]. Human DCs express CB_1 and CB_2 receptors and also produce AEA and 2-AG [146], suggesting potential effects on the TME. CB_2 has been recently reported to influence dendritic cell maturation [230]. Another innate immune cell population with cannabinoid receptor expression is NK cells [16], which are well linked to tumor regression [231]. Cannabinoids such as Δ^9-THC are able to suppress NK activity [232] which may involve both CB_1 and CB_2 receptors [233].

To summarize, regulation of T cells, macrophages and dendritic cells by endocannabinoids and MGL is a potential mechanism by the ECS to control tumor growth. This could be primarily achieved by CB_2 activity, although CB_1 and cannabinoid receptor-independent mechanisms may also be involved (rev in [234]). The role of the ECS in neutrophils awaits exploration. For additional reading about cannabinoids and immunity (not only related to cancer), the reader is referred to the following reviews: [206,235,236].

7. Potential Role of Endocannabinoids in the Tumor Microenvironment

Although alterations in endocannabinoid levels have been demonstrated in tumors vs. non-neoplastic tissue [237], a clear understanding of how endocannabinoids impact the immune TME is hardly known. As to the role of endocannabinoids within the TME, their actions are likely dependent on their

local concentration, status of immune cell activation and expression levels of cannabinoid receptors. As shown by Sailler et al. [237] and by our own studies [223], endocannabinoid profiles are deranged in tumors and plasma of cancer patients in comparison to control tissue/plasma. In many tumors, levels of 2-AG and AEA are increased, such as in brain, intestinal and gynecological tumors (summarized in [20]), whereas one study shows increases in 2-AG, but decreases in AEA, PEA and OEA in mice with local tumor growth [237], suggesting a consistent role of endocannabinoids in cancer only for 2-AG.

As in the case of immune cells, endocannabinoids were shown to suppress proliferation of T cells, migration of $CD8^+$ cells and neutrophils, and the release of proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-17 and IFN-γ) from $CD8^+$, $CD4^+$, and dendritic cells and from macrophages (summarized and discussed in [146]). 2-AG has been described to cause migration of human peripheral blood monocytes, neutrophils, eosinophils, NK cells, mouse dendritic cells, and B cells [156–158,160,165,167,169]. In a pancreatic tumor model, 2-AG caused an increase in MDSCs, indicating immunosuppressive effects in vivo [29]. Adding to the endocannabinoids' complex behavior, their effects may not be always mediated by cannabinoid receptors: Chouinard et al. demonstrated that 2-AG activated human neutrophils independently of cannabinoid receptors by a mechanism that includes 2-AG hydrolysis, de novo LTB_4 biosynthesis, and an autocrine activation loop involving LTB_4 receptor 1 [168]. 2-AG may also serve as a substrate for cyclo-oxygenase (COX)-catalyzing PGE_2 production (e.g., via liberation of arachidonic acid by other hydrolases such as MGL), which in turn could modulate the action of CB_2 [238]. In the TME, immune cells like macrophages could be a source of endocannabinoids. Their synthesis may even be stimulated by endocannabinoids themselves [185]. Macrophages such as mouse peritoneal macrophages [187], J774 [182] and P388D1 macrophages [188] were all shown to synthesize 2-AG. Furthermore, AEA is formed in macrophages [239] and was shown to maintain the level of regulatory macrophages (high expression of chemokine receptor CX3CR1) in gut tissue [240], supporting the idea that also in the TME, macrophage behavior is likely regulated by endocannabinoids.

It should be kept in mind that many effects of cannabinoids have been measured by in vitro experiments, and the effects were dependent on the concentration of the (endo)cannabinoids applied. Berdyshev and colleagues demonstrated that while AEA diminished IL-6 and IL-8 production in PBMCs at low nanomolar concentrations (3–30 nM), these effects disappeared after increasing the concentration [241]. Likewise, cannabinoids such as Δ^9-THC, and the synthetic cannabinoid receptor ligands WIN 55212-2 and CP55,940, enhanced proliferation of human B cells at low (nM range), but not at high concentrations (10 μM) [242]. Since tissue concentrations of endocannabinoids lie in the nM range/g tissue (e.g., in colon mucosal tissue; [243]), effects seen with high μM concentrations in vitro may not be relevant for in vivo (patho)-physiology of immune cells. In vivo actions of endocannabinoids in the TME, therefore, could differ from those known from in vitro experiments.

To summarize, plenty of data show that endocannabinoids concentration-dependently influence immune cell behavior, a situation that may likely occur in the TME. The potential role of endocannabinoids in primary tumors and metastases has also been recently discussed elsewhere [244].

8. Cannabinoids as Potential Drugs That Affect the Tumor Microenvironment and Tumor Growth

The fact that cannabinoids can influence the immune cell behavior and, therefore, also the immune cell infiltrate of tumors (and hence tumor growth), naturally raises the suggestion that cannabinoid-based drugs could be used as anti-tumor agents alone or in combination with immunotherapy. Cannabinoid receptors are expressed on immune cells and cannabinoids may, therefore, modulate anti-tumor immune responses. Presently, cannabis and synthetic cannabinoids, such as nabilone, are mainly used as antiemetics during chemotherapy. Although preclinical models have demonstrated potent anticancer effects [245], sufficient evidence for the use of cannabis in cancer only exists in palliative care but not in anti-tumor therapy [246]. Δ^9-THC has shown immunosuppressive effects and causes an increase in tumor growth in a breast cancer model [247], suggesting that the immune system does not favorably respond to cannabinoid treatment in cancer. Clinical studies also report

that cannabis use decreased the response rate in cancer patients during immunotherapy with the PD-1 inhibitor nivolumab [248], and correlated with poor clinical outcome [249]. These results may speak against cannabinoids as add-ons in immunotherapy. However, it is still unclear how pharmacological inhibition of cannabinoid receptors or other targets of the ECS may affect immunotherapy in preclinical models of cancer.

9. Conclusions

In vitro studies have demonstrated that the behavior of immune cells is regulated by (endo)cannabinoids and other components of the ECS, indicating that the ECS effectively influences the immune landscape of tumors. This has been now supported by in vivo studies highlighting the importance of macrophages and MDSCs of the TME in the actions of the ECS on tumor growth (e.g., [29,34,35]). ECS components of the TME could be responsible for the fate of tumor growth by working synergistically, independently or in an opposing manner. Knowledge on the role of the ECS in the regulation of the "tumor immune microenvironment" may be important in establishing a more effective anti-neoplastic therapy.

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Monoacylglycerol lipase deficiency in the tumor microenvironment slows tumor growth in non-small cell lung cancer

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ABSTRACT

Monoacylglycerol lipase (MGL) expressed in cancer cells influences cancer pathogenesis but the role of MGL in the tumor microenvironment (TME) is less known. Using a syngeneic tumor model with KP cells ($Kras^{LSL\text{-}G12D}/p53^{fl/fl}$; from mouse lung adenocarcinoma), we investigated whether TME-expressed MGL plays a role in tumor growth of non-small cell lung cancer (NSCLC).

In sections of human and experimental NSCLC, MGL was found in tumor cells and various cells of the TME including macrophages and stromal cells. Mice treated with the MGL inhibitor JZL184 as well as MGL knock-out (KO) mice exhibited a lower tumor burden than the controls. The reduction in tumor growth was accompanied by an increased number of $CD8^+$ T cells and eosinophils. Naïve $CD8^+$ T cells showed a shift toward more effector cells in MGL KOs and an increased expression of granzyme-B and interferon-γ, indicative of enhanced tumoricidal activity. 2-arachidonoyl glycerol (2-AG) was increased in tumors of MGL KO mice, and dose-dependently induced differentiation and migration of $CD8^+$ T cells as well as migration and activation of eosinophils *in vitro*.

Our results suggest that next to cancer cell-derived MGL, TME cells expressing MGL are responsible for maintaining a pro-tumorigenic environment in tumors of NSCLC.

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Introduction

Monoacylglycerol lipase (MGL) was characterized several decades ago as the major enzyme that hydrolyzes monoglycerides into glycerol and fatty acids (rev. in[1]). One of these monoglycerides is 2-arachidonoyl glycerol (2-AG),[2] which has been also identified as an endocannabinoid (rev. in[3]). 2-AG is a full agonist at cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors, thereby causing cannabimimetic effects.[4] MGL contributes to many (patho)physiological conditions, such as pain, food intake, stress, metabolic disorders, addiction, inflammation, and cancer (rev. in[1]). Cannabinoid receptor signaling is terminated through degradation of 2-AG by MGL, which therefore plays, aside from its lipolytic function, a major role in endocannabinoid metabolism.[3] It accounts for around 85% of brain 2-AG hydrolase activity while the remaining part is degraded by enzymes like α/β-hydrolase domain containing (ABHD) 6 and 12.[5] 2-AG, CB_1/CB_2 and MGL are a chain of key regulators that operate cooperatively within the endocannabinoid system (ECS). Notably, pharmacological inhibition or genetic deletion of MGL results in the accumulation of endogenous 2-AG, but not of arachidonoyl ethanolamide (anandamide; AEA), another well-described endocannabinoid.[6–9] In addition, MGL provides a large pool of arachidonic acid (AA), from which pro-inflammatory prostaglandins may be generated.[10] This indicates that besides 2-AG, MGL regulates the availability of AA.[8,11]

Apart from metabolizing 2-AG, MGL is known to break down other MGs with different fatty acid chain length and saturation,[12] as well as prostaglandin glycerol esters.[13] The enzyme plays an important role in the intestine where reduced triglyceride secretion after oral fat challenge has been described in MGL deficient mice.[14] MGL also acts as a fatty acid ethyl ester hydrolase and thus may counteract the formation of potentially toxic byproducts of ethanol metabolism.[15]

MGL has lately emerged as an interesting pharmacological target for the treatment of inflammatory diseases and cancer.[16,17] During carcinogenesis, tumor cells shift their energy metabolism toward glycolysis and lipid synthesis.[18] Fatty acids support cancer progression, e.g., by providing building blocks for new membranes. They are also needed for the generation of bioactive lipids that function as signaling molecules.[18] Studies have shown that increased MGL expression/activity in cancer cells drives tumorigenesis through remodeling of fatty acids,[19] suggesting a tumor-promoting role for cancer cell-derived MGL. In keeping with MGL's pro-tumorigenic role, MGL knockdown or treatment with the

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MGL inhibitor JZL184 results in reduced proliferation/invasion and increased apoptosis in colorectal cancer (CRC) cell lines.[20,21] Furthermore, treatment with JZL184 reduces metastasis of A549 lung cancer cells in nude mice.[22] High levels of MGL were seen in human melanoma and endometrial cancer samples.[23,24] Other studies showed reduced MGL levels in human samples of CRC[25] and squamous cell carcinoma.[26] In one of these studies, overexpression of MGL in HCT116 CRC cells showed reduced colony formation.[25] In old MGL KO mice, tumors supposedly develop spontaneously, particularly in the lung,[27] suggesting that MGL may rather act as a tumor-suppressor. However, MGL has been considered an unfavorable prognostic factor in primary gastrointestinal stromal tumors[28] and lung adenocarcinoma.[29] At the same time, in pancreatic cancer, no correlation of MGL with survival was detected,[30] indicating that expression of MGL and correlation with tumor aggressiveness in human cancers is still unclear and may depend on the tumor entity and context.

In contrast to our knowledge of the role of MGL in tumor cells, not much is known about the presence and function of MGL in the immune and stromal cells of the tumor microenvironment (TME), in particular, as to how TME-derived MGL influences tumorigenesis. Immune cells express cannabinoid receptors, and they generate/degrade and respond to endocannabinoids, virtually forming an "immune-endocannabinoid system" (rev. in[31,32]). MGL has been detected in macrophages/monocytes and eosinophils (rev. in[32]), and a recent study suggests that MGL is present in tumor-associated macrophages (TAMs) that regulate tumor progression in mouse models of CRC.[33] Using RNAScope® in situ hybridization (ISH), we recently showed that T cells ($CD3^+$) and macrophages of mouse intestines express MGL.[34]

$CD8^+$ T cells and eosinophils represent two important anti-tumorigenic immune cell populations in the TME. One of the main roles for $CD8^+$ T cells in the TME is the killing of tumor cells. $CD8^+$ cells release cytotoxic granzymes and perforin[35] via recognition of tumor antigen presented by MHC-1.[36] In line with their tumor cell cytotoxicity, increased presence of $CD8^+$ T cells was reported to correlate with a better outcome in several types of cancer, such as breast[37] and colorectal cancer.[38] The role of eosinophils in the TME is less clear. Although tumor eosinophilia has been correlated with lower tumor progression, the opposite has been also reported.[39] Similar to $CD8^+$ T cells, their antitumorigenic properties include the degranulation of cytotoxic molecules[40,41] and the recruitment of anti-tumorigenic leukocytes.[42] Because of the presence of cannabinoid receptors in these cells (reviewed in[32]), both $CD8^+$ T cells and eosinophils can respond to 2-AG. Low MGL expression in tumor tissue could increase 2-AG levels and locally affect the migration and proliferation of these cells; however, this has not been clarified yet.

As the major regulator of 2-AG levels, MGL is critical for CB_1 receptor (de)sensitization and the endocannabinoid tone.[43] By inhibiting MGL, endogenous 2-AG levels increase in a tissue-specific pattern.[6] Tumors also exhibit elevated 2-AG levels, as observed in endometrial cancer.[44] Amongst various endocannabinoids and endocannabinoid-like lipids, only 2-AG has consistently been shown to be increased in experimentally induced tumors (current study).[45,46] Our own work[47] and a study by Sailler et al.[46] demonstrate that 2-AG levels are increased in plasma of patients with CRC and other types of cancer. Both pro- and anti-inflammatory effects in immune cells have been reported for 2-AG,[31] therefore, elevated levels of 2-AG in tumors could impact immune cell behavior in the TME in opposite ways. Effects of 2-AG on immune cells usually involve cannabinoid receptors, mostly CB_2 (rev. in[31,48]), although CB_1 or cannabinoid receptor independent effects, such as leukotriene biosynthesis and LTB4 activation might be involved.[49,50]

In the current study, we investigated the role of MGL in the development of NSCLC, with a focus on MGL in the TME. We aimed to identify a potential influence of MGL-expressing TME cells on tumor growth, and on changes in the immune cell landscape of tumors. In particular, we were interested where in the TME MGL is expressed. The TME, specifically the infiltrated immune cell profile, critically determines tumor progression.[51] We used $MGL^{-/-}$ knockout (MGL KO) and wild type (WT) mice in a syngeneic NSCLC model in which mice were subcutaneously (s.c.) injected with KP cells ($Kras^{LSL\text{-}G12D}$, $p53^{fl/fl}$; established from mouse lung adenocarcinoma) to induce the development of a s.c. tumor. We show that MGL KO mice and mice treated with the MGL inhibitor JZl184 have a lower tumor burden than the WT littermates or vehicle-treated mice, respectively, indicating a pro-tumorigenic role of MGL in NSCLC.

Materials and methods

Animal studies and cell culture

All animal experiments were performed in the animal facilities of the Medical University of Graz. C57BL/6J mice were purchased from Charles River. Approval for animal experimental protocols was granted by the Austrian Federal Ministry of Science and Research (protocol number: BMBWF-66.010/0041-V/3b/2018).

MGL KO mice were a gift from Dr. R. Zimmermann[9] from the University of Graz and bred in-house together with wild-type (WT) littermates. The murine KP cell line was isolated from a lung adenocarcinoma of a $Kras^{LSL\text{-}G12D}/p53^{fl/fl}$ mouse at the Fred Hutchinson Cancer Center (Seattle, WA, USA) after intratracheal administration of adenoviral Cre recombinase as described before.[52] The cell line was generously provided by Dr. McGarry Houghton. KP cells were maintained in DMEM with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (P/S, PAA Laboratories) at 37°C and 5% CO_2 in a humidified atmosphere. For the generation of a MGL-overexpressing KP cell line, parental KP cells were transduced with a lentivirus carrying a MGL-CFP-Puro-cassette (KP_MGL) or a control cassette (KP_ctrl), which were kindly provided by Dr. C. Heier from the University of Graz.[15] Selection for positive single clones was performed with puromycin (1 µg/ml, Thermo Fisher, A1113803) and confirmed using flow cytometry. Primary human cancer-associated fibroblasts were kindly provided by Dr. K. Leithner from the Medical University of Graz[53] and cultured in DMEM supplemented with 10% FBS and 1% P/S. Cells were starved overnight with serum-free media and 1 or 5 ng/ml TGF-β (Bio-Techne, 7754-BH-005) for 3 days and were used for differentiation.

Mouse primary dermal fibroblasts were cultured according to the manufacturer's protocol (Pelobiotech, Martinsried, Germany).

Murine tumor models

Parental KP cells (0.5×10^6) were injected (s.c.) into the right flank of mice. For pharmacological inhibition of MGL, tumor-bearing C57BL/6J wild-type mice were intraperitoneally (i.p.) treated with 16 mg/kg JZL184 (MGL inhibitor, Cayman), once daily, starting one day prior to the injection of tumor cells. For the experiments with MGL-overexpressing cells, either 0.5×10^6 KP _MGL or KP _ctrl cells were injected (s.c.) into the right flank of C57BL/6J wild-type mice. Tumor growth was monitored during the course of the experiments twice per week using a caliper. Mice were sacrificed after about two weeks, and tumors were subsequently collected, weighted, and measured with a caliper *ex vivo*. Tumor volume was calculated according to the following formula: v = length x width x height x π/6.[40]

Single-cell suspensions

The preparation of single-cell suspensions from tumors was performed as previously described.[40] Using a scalpel, tumors were minced, and digested with DNase I (160 U/ml; Worthington) and collagenase (4.5 U/ml; Worthington) for 15 minutes at 37°C while rotating at 1000 rpm. After brief vortexing, samples were incubated for 10 min before they were passed through a 40 µm cell strainer, washed in PBS +2% FBS, counted, and used for antigen staining.

Flow cytometric phenotyping of immune cell populations

First, single cell suspensions were incubated for 20 min in Fixable Viability Dye (FVD) eFluor™ 780 (eBioscience) in the dark to exclude dead cells. After adding 1 µg TruStain™ FcX (Biolegend), immunostaining was performed on ice for 30 min (protected from light) with the following antibodies: CD45-AF700 (# 103,128), CD45-BV785 (# 103,149), Ly6C-APC (# 128,015), Ly6C-FITC (# 128,005), Ly6G-PE /Dazzle594 (# 127,648), CD11 c-BV605 (# 117,334), CD8-PerCPCy5.5 (# 100,734), CD63-PE (# 143,903), PD1-APC (# 135,210), CD62L-BV605 (# 104,438), NKp46-BV510 (# 137,623), CD4-PECy7 (# 100,422), CD4-APC (# 100,516), CD19-PECy7 (# 115,520), CD44-FITC (# 103,005), PD1-BV421 (#135,221), CD62L-BV421 (# 104,436) (all antibodies from Biolegend), and CD11b-BUV737 (# 612,801), F4/80-PE (# 565,410), Siglec-F-PerCP5.5 (# 565,526), F4/80-BUV395 (# 565,614), CD3-BUV395 (# 563,565), CD4-BUV496 (# 564,667), CD44-BUV737 (# 612,799), Siglec-F-PE (# 562,068) (all antibodies from BD Biosciences). For detection of changes in IFN-γ and granzyme-B expression, single-cell suspensions of tumors or spleens (2×10^6 cells per well) were seeded into 96-well U-bottomed plates with RPMI containing 10% FBS, 1% P/S, and GolgiStop (1.5 µl/ml, BD Biosciences) and left for 4 h at 37°C. During that time, they were stimulated with PMA (100 ng/ml, Sigma Aldrich) and ionomycin (1 µg/ml, Sigma Aldrich) or kept without stimulation. After that, surface staining was performed with CD45-AF700, CD3-BUV395 and CD8-PerCP5.5, followed by intracellular labeling (BD Cytofix/Cytoperm™ Kit) with IFN-γ-PECF594 (BD Biosciences, # 562,303) and granzyme-B-AF647 (# 515,406). After staining, cells were washed and fixed using IC Fixation Buffer (eBioscience). Samples were either analyzed on a BD LSRFortessa™ or BD Canto™ flow cytometer with FACSDiva software (BD Biosciences). Analyses and compensation were performed with Flowjo software (TreeStar). Fluorescence minus-one-samples were used to define gates. See *Supplementary figure S1* for gating strategies.

RNA extraction and qRT-PCR

RNA extraction from tissue was carried out in TRIzol (Life Technologies). For RNA extraction from cultured cells, RNeasy Kit (Qiagen) was used. Samples were treated with either a DNA-free DNA Removal Kit (Invitrogen) or RNase-Free DNase set (Qiagen). One µg of RNA was reversely transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantification of gene expression by RT-qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were acquired from Eurofins (*Supplementary table 1*) or Bio-Rad (*Supplementary table 2*). ΔCq- and ΔΔCq-methods were used for the assessment of relative gene expression.[54]

Differentiation of bone marrow-derived eosinophils (BMDEs)

Bone marrow was isolated from C57BL/6J wild-type mice, and eosinophils were differentiated as previously described.[40] In short, after lysis of erythrocytes with ddH_2O and 10xPBS, cells were cultured in BMDE-RPMI, i.e. RPMI+20% HyClone FBS (GE Healthcare; # 10,309,433), 25 mM HEPES (Thermo Fisher; # 15,630–080), 1xnon-essential amino acids (Thermo Fisher; # 11,140–035), 1 mM sodium pyruvate (Thermo Fisher; #11,360–039), 1% P/S and 50 µM beta-mercaptoethanol (Sigma-Aldrich; M3148) supplemented with 100 ng/ml FLT3L (PreproTech; # 250–31 L) and 100 ng/ml stem cell factor (PreproTech; # 250–03) for four days at 37°C and 5% CO_2. After that, the medium was changed to BMDE-RPMI supplemented only with 10 ng/ml IL-5 (Bio-Techne). Every fourth day, cells were transferred to a new flask, and a fresh medium was added every other day. Fully differentiated BMDEs were used for assays on day 14.

Ca^{2+} flux in eosinophils

BMDEs were treated with 2 µM of Fluo-3 AM in the presence of 0.02% pluronic F-127 for 1 hr at room temperature and protected from light.[55] Then, 1 µM of the CB_2 antagonist SR144528 or EtOH as control was added for the last 10 min. BMDEs were subjected to baseline measurement for 60 sec and additionally stimulated with increasing concentrations of 2-AG (Tocris) or DMSO (vehicle). Changes in intracellular Ca^{2+} were determined as an increase in the 530/30 nm channel on a BDCalibur flow cytometer.

Isolation of lymphocytes from spleen

$CD3^+$ and $CD8^+$ T cells were isolated from spleens of MGL KO mice and their WT littermates with EasySep™ mouse $CD8^+$ T cell/T cell isolation kit according to the manufacturer's protocol (Stemcell).

BMDE and $CD8^+$ T cell migration assay

Migration assays were performed using 5 µm Transwell plates (Corning), as previously described.[56] In brief, BMDEs and negatively isolated murine $CD8^+$ T cells were seeded in the upper well (at 1×10^5 or 1.5×10^5 cells per well, respectively), and increasing concentrations of 2-AG were used as a chemoattractant for 1 or 4 h at 37°C, respectively. DMSO was used as vehicle. SR144528 or EtOH (control) was used as a pretreatment (1 µM, 10 min at room temperature). CCL19 (Biolegend) was used as a positive control for $CD8^+$ T cell migration. Enumeration of cells migrating to the lower well was performed on a BDCanto™ flow cytometer (BD Biosciences).

T cell differentiation and proliferation assay

Isolated murine T cells were seeded into 96-well U-bottom plates (1.5×10^5 cells per well, pre-coated with 5 µg/ml ULTRA-LEAF™ anti-mouse CD3 antibody (Biolegend)) with X–VIVO™ 15 medium (Szabo-Scandic) containing 1% P/S, 2 nM L-glutamine, 50 µM β-mercaptoethanol, 25 mM HEPES, 50 U/ml mIL-2, 1xnon-essential amino acids (Thermo Fisher; # 11,140–035), 1 mM sodium pyruvate (Thermo Fisher; # 11,360–039), and ULTRA-LEAF™ anti-mouse CD28 antibody 1 µg/ml (Biolegend). T cells were then treated with different concentrations of 2-AG following pre-treatment with 1 µM SR144528 or vehicle for 30 min. To determine T cell proliferation, cells were pre-loaded with 5 µM eFluor™ 450 cell proliferation dye (Invitrogen) for 10 min at 37°C. After 4 days of incubation at 37°C, T cells were harvested and staining was performed as described above.

In situ hybridization (ISH) and immunofluorescence

Tumors were fixed in acid-free phosphate-buffered 10% formaldehyde solution (Roti®-Histofix 10%, pH7) for 16–24 h at room temperature, and further processed for paraffin embedding according to standard procedures. Tissue was cut in 5 µm sections, baked at 60°C for 1 hr, de-waxed and rehydrated. ISH was performed according to the manufacturer's protocol and as recently published.[34] In brief, three ZZ probes for MGL (targeting bases 703–849 of NM_001166251.1) (BaseScope™ RED kit; Advanced Cell Diagnostics [ACD]) were used to detect the corresponding mRNAs in tumors. Sections with tumor tissue were treated with H_2O_2 for 10 min and target retrieval was performed using the Brown FS3000 food steamer for 15 min. Each step was followed by washes in distilled water. The sections were then digested with Protease IV at 40°C for 20 min, washed, followed by incubation with the corresponding probes at 40°C for 2 h and stained using FastRed. Samples from MGL KO and WT mice were put on one slide for comparison. MGL KO mice lacked expression of MGL outside of the engrafted KP tumor cells. The specificity of the MGL probe is also published elsewhere.[34]

ISH and immunofluorescence were performed on 4 µm sections of the human lung adenocarcinoma formalin-fixed paraffin-embedded samples obtained from the Biobank of the Medical University of Graz. mRNA was detected using an anti-human MGL probe (targeting bases 566–1530 of NM_007283.6 (RNAScope 2.5 HD Assay-RED kit, ACD; cat. # 539,151)). Ethical approval was obtained from the Institutional Review Board of the Medical University of Graz (EK-numbers: 30–105 ex 17/18). All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects involved in the study.

Antibodies against cytokeratin (1:200; Dako # ZO0622), F4/80 (1:500; Cell Signaling # 70,076) or $CD163^+$ (1:500; Abcam # ab182422), $CD3^+$ (1:500; Novus # NB6001441SS), CD11b (1:100; Novus # NB11089474), fibroblast-specific protein (FSP) (1:100; Millipore #72,274), von Willebrand factor (vWF) (1:400; Abcam # ab6994), α-SMA (1:100; Abcam # ab5694), were used to determine tumor cells and cell types of the immune/stromal TME co-localizing with MGL mRNAs. After ISH, tissue sections were first blocked in 0.1 M PBS containing 0.3% Triton X-100 and 5% goat serum (Sigma-Aldrich). Primary antibodies were applied in 0.1 M PBS containing 0.3% Triton X-100 and 1% goat serum over night at 4°C. As the second antibody, Alexa Fluor® 488-labeled goat anti-rabbit IgG (1:500; Jackson Immuno Research; #111-546-144) was used. Sections were also equally processed without primary antibodies as a negative control. Afterward, sections were mounted with Vectashield® (containing DAPI) (Vector Laboratories) and images were taken by an Olympus IX70 fluorescence microscope (Olympus) connected with a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Japan). Images were processed with Olympus CellSense® 1.17 imaging software (Olympus). The contrast, brightness and color balance of images were adjusted using Corel Photo Paint®.

LC-MS analytics of endocannabinoids and other lipids

Endocannabinoids, endocannabinoid-like substances, DGs, TGs and other lipids from mouse tumor and adjacent white adipose tissue were determined using liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) and high-resolution mass spectrometry (LC-HRMS), respectively. Details about the used equipment, materials, method parameters and a full list of analyzed analytes can be found in the *Supplementary materials S2 (LC-MS analytics)*.

Tissue was homogenized prior to lipid extraction using wet grinding in a Precellys 24 (Bertin Instruments, Montigny-le-Bretonneux, France) at 10°C. The tissue homogenates had a concentration of 0.05 mg/µL in ethanol:water (1:3, v/v), and 10 zirconium dioxide balls were added for the grinding. For quantification of endocannabinoids and related substances,

tissue homogenates equaling 2 mg of tissue and a 1:50 dilution in ethanol:water (1:3, v/v) were extracted after filling up the sample volume to 200 µL with ethanol:water (1:3, v/v). Quantitation of endocannabinoids and related compounds was achieved as described previously with a shortened gradient and reduced source temperature of 400°C to enhance sensitivity of 2-oleoyl glycerol (2-OG).[57] Due to the shortened gradient, OEA and its isomer vaccenic acid ethanolamide (VEA) were not separated and were, therefore, quantified as a sum parameter.

1-arachidonoyl glycerol (1-AG), 2-AG, arachidonoyl ethanolamide (AEA), oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA) and 2-OG were extracted from 200 µL sample volume using liquid-liquid-extraction by adding 20 µL of internal standards in acetonitrile, 20 µL acetonitrile and 400 µL ethyl acetate: hexane (9:1, v/v). After mixing and centrifugation at 4°C for 3 min, the upper organic layer was transferred and dried under a nitrogen stream at 45°C. Before analysis, samples were dissolved in 50 µL acetonitrile. The LC-MS/MS measurement included separation on an Agilent 1290 Infinity II UHPLC system with an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm, Waters, Eschborn, Germany) and measurement on a QTrap 6500+ (Sciex, Darmstadt, Germany) using an ESI Turbo-V-source with negative ion source voltage. Data were acquired using Analyst software v1.7.1 and further analyzed with MultiQuant software v 3.0.2. Acceptance criteria and quality assurance measures were applied as described previously.[57] The lower and upper limits of quantification for all analytes can be found in the *Supplementary materials S2*.

The lipid screening using LC-HRMS was performed as described previously with a shortened gradient, which is further described in the *Supplementary materials S2*.[58] Additionally, oleic acid was quantified within this analysis. Lipids were extracted using a modified MTBE extraction protocol.[59] A volume of 10 µL tissue homogenate (≡ 0.5 mg tissue) was mixed with 75 µL of internal standards in methanol, 10 µL of oleic acid-d9 in methanol, 250 µL MTBE and 60 µL 50 mM ammonium formate. The mixture was vortexed for 1 min and centrifuged for 5 min at 20,000 g at room temperature. After transfer of the upper organic phase, the aqueous phase was re-extracted with 100 µL of a mixture of MTBE: methanol: water (10:3:2.5, v/v/v, upper phase). The combined organic phases were dried under a stream of nitrogen at 45°C and reconstituted in 100 µL methanol before LC-HRMS analysis. For the calibration standards and quality control samples for oleic acid, 20 µL of ethanol:water 1:3 (v/v) was mixed with 20 µl standard/qc solution in methanol containing 0,1% BHT, 55 µL methanol, 10 µl oleic acid-d9 in methanol, 250 µL MTBE and 60 µL 50 mM ammonium formate and extracted as described above.

The measurement was performed using a Vanquish Horizon UHPLC system coupled to an Exploris 480 (both Thermo Fisher Scientific, Dreieich, Germany) applying a Zorbax RRHD Eclipse Plus C8 column (1.8 µm 50 × 2.1 mm ID, Agilent, Waldbronn, Germany). Both ionization modes were used with a scan range from 180 to 1500 m/z and a mass resolution of 120.000 combined with data-dependent acquisition at 15.000 mass resolution with a full scan every 0.6 s. For verification of the system stability, the first twelve samples were pooled and replicates injected at the start, at the end of a run as well as after every 10th sample. For data acquisition and peak integration, XCalibur software v4.4 and TraceFinder software v5.1 were used with a mass tolerance of 5 ppm.

In a semi-targeted approach, 318 lipids were evaluated and results were normalized to one internal standard per lipid class. These lipids were identified using exact mass ± 5 ppm, isotope ratio and comparison of MS/MS fragmentation pattern with LipidBlast database.[60] A relative standard deviation below 20% for the quality control samples was used as acceptance criteria for the lipid screening. Oleic acid was quantified in negative ionization mode full scan as hydrogen-loss and the same quality assurance and acceptance criteria as for the endocannabinoids were applied.[57]

Statistical analysis

GraphPad Prism 6.1 (GraphPad® Software) was used to perform statistical analyses for *in vitro* and *in vivo* experiments. Gaussian distribution was tested by using Shapiro-Wilk normality test. Statistically significant differences between two experimental groups were determined using unpaired student's *t*-tests, multiple *t*-tests or two-way ANOVA with the indicated *post hoc* test for corrections of multiple comparisons. To compare three or more groups, one-way ANOVA was used with the indicated post hoc test for corrections of multiple comparisons. Statistical analysis for not normally distributed data was performed using non-parametric Mann-Whitney tests and Kruskal-Wallis test with Dunn's multiple comparison test. Correlations between tumor weight/volume and MGL, ABHD6 or 12 expressions were determined using Pearson's correlation coefficient (r) and Spearman's correlation coefficient *rho* (r_s).

Lipid species data was analyzed in R (Version 4.0.3) using Welch's t-test, and plots were drawn with the pheatmap-package (Raivo Kolde, 2019. pheatmap: Pretty Heatmaps. R package version 1.0.12. *https://CRAN.R-project.org/package=pheatmap*). Colors indicate the up- (red) or down (blue) regulation of each lipid.

P values < .05 were considered significant and denoted with 1, 2 or 3 asterisks when lower than 0.05, 0.01 or 0.001, respectively.

Results

MGL-deficiency in cells of the TME inhibits tumor growth in a syngeneic NSCLC model

In recent publications, reduced lung cancer cell invasiveness was detected after treatment with an MGL inhibitor.[22,29] We, therefore, treated KP cell tumor bearing C57BL/6J mice with the MGL inhibitor JZL184 (or vehicle) first to investigate whether systemic blockade of MGL affects primary tumor growth (Figure 1a). As a result, the inhibitor slowed tumor progression (Figure 1b) and caused a significant reduction in tumor weight and volume (Figure 1c). To validate whether JZL184 truly inhibited MGL activity, we performed a monoacylglycerol hydrolase (MGH)

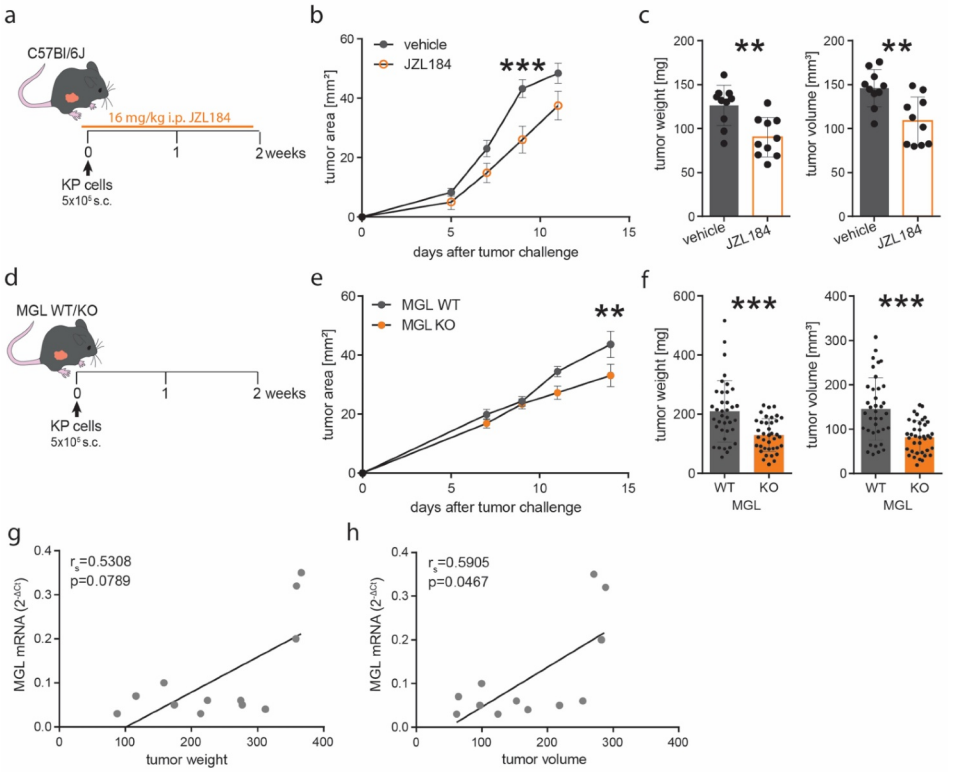

Figure 1. Pharmacological inhibition of MGL and genetic MGL deficiency in cells of the TME inhibit tumor growth in a model of non-small cell lung cancer. (**a**) C57BL/6J wild-type mice were injected subcutaneously (s.c.) with 5×10^5 KP lung adenocarcinoma cells (time point 0) and tumors were allowed to grow for 12 days. Mice were additionally injected intraperitoneally (i.p.) either with 16 mg/kg of the MGL inhibitor JZL184 (or vehicle) once daily, starting the day before tumor cell injections. (**b**) Tumor development was monitored over the course of the experiment. Data indicate means ± SEM. One representative experiment is shown. n = 10. (**c**) *Ex vivo* tumor weight and volume were evaluated at the end of the experiment. One representative experiment is shown. Data indicate means + SEM. n = 10. (**d**) MGL wild type (WT) and knockout (KO) mice were injected s.c. with 5×10^5 KP lung adenocarcinoma cells (time point 0) and tumors were allowed to grow for 15 days. (**e**) Tumor development was monitored during the course of the experiment. Data indicate means ± SEM. One representative experiment is shown. n = 9–10. (**f**) After 15 days, mice were sacrificed, and tumor weight and volume were measured *ex vivo*. Data indicate means + SEM and were pooled from four independent experiments. n = 37–38. Correlations between MGL mRNA expression and tumor weight (**g**) or volume (**h**). Two representative experiments were pooled. n = 12. Statistical differences were assessed using two-way ANOVA with Tukey's multiple comparison test, student's *t*-test or Mann-Whitney test. Correlation was determined by using Spearman's correlation coefficient rho (r_s). $^{**}p < .01$, $^{***}p < .001$

activity assay in protein lysates of the liver from vehicle- and JZL184-treated mice, demonstrating the systemically inhibiting effect of JZL184 on MGL activity (*Supplementary figure S3a*). JZL184 treatment had no direct effect on tumor cells and did not influence tumor cell viability (evaluated as $CD45^-$ cells; *Supplementary figure S3b*) or proliferation (*Supplementary figure S3c and d*) *in vivo*.

As the role of MGL has not yet been investigated in the TME of NSCLC, we aimed to find out whether tumor infiltrating cells that were deficient of MGL (thus creating a MGL deficient TME) would be sufficient to reduce tumor growth. We injected KP cells into the flank of MGL KO and WT mice (Figure 1d). The growth of tumors was monitored over 2 weeks and similar to the effect of JZL184, we found reduced tumor growth in the MGL KO vs. WT mice (Figure 1e). *Ex vivo* analysis of tumor weight and volume showed an approximate 40% reduction of tumor size in MGL KO mice (Figure 1f). mRNA expression of MGL in tumors from WT mice positively correlated with tumor size (Figure 1 g, h). mRNA expression of other monoglyceride hydrolases like ABHD6 (recently suggested as a tumor-driver in NSCLC[61]) and ABHD12 showed no significant correlations (*Supplementary figures S3e-h*).

MGL deficiency in cells of the TME favors an anti-tumorigenic immune cell profile

We next investigated whether the reduced tumor size that we observed in MGL KO mice was accompanied by changes in the immune cell profile of the TME (Figure 2a-g). In comparison to WT mice, flow cytometry of tumor-infiltrating leukocytes in the MGL KO mice revealed significant increases in the number of eosinophils (Figure 2c) and $CD8^+$ T cells (Figure 2e), indicating a potential shift to an anti-tumorigenic environment. However, no differences were detected in $CD4^+$ T, overall T, NK, NKT and B cells (Figure 2d). In WTs, the relative abundance of naïve $CD4^+$ and $CD8^+$ T cells were higher than in KO mice (Figure 2f), while in KO mice, the relative abundance of effector $CD8^+$ T cells was higher than in WTs (Figure 2g). The expression of the inhibitory checkpoint receptor PD-1 on tumor-infiltrating $CD4^+$ and $CD8^+$ T cells was increased in tumors from MGL KO mice suggesting increased immune activity of these cells (Figure 2h). Expression levels of granzyme-B (Figure 2i, j) and IFN-γ (Figure 2k, l) were increased in tumor-infiltrating $CD8^+$ T cells of MGL KO vs. WT mice, indicating enhanced tumoricidal activity of $CD8^+$ T cells in MGL KO mice.

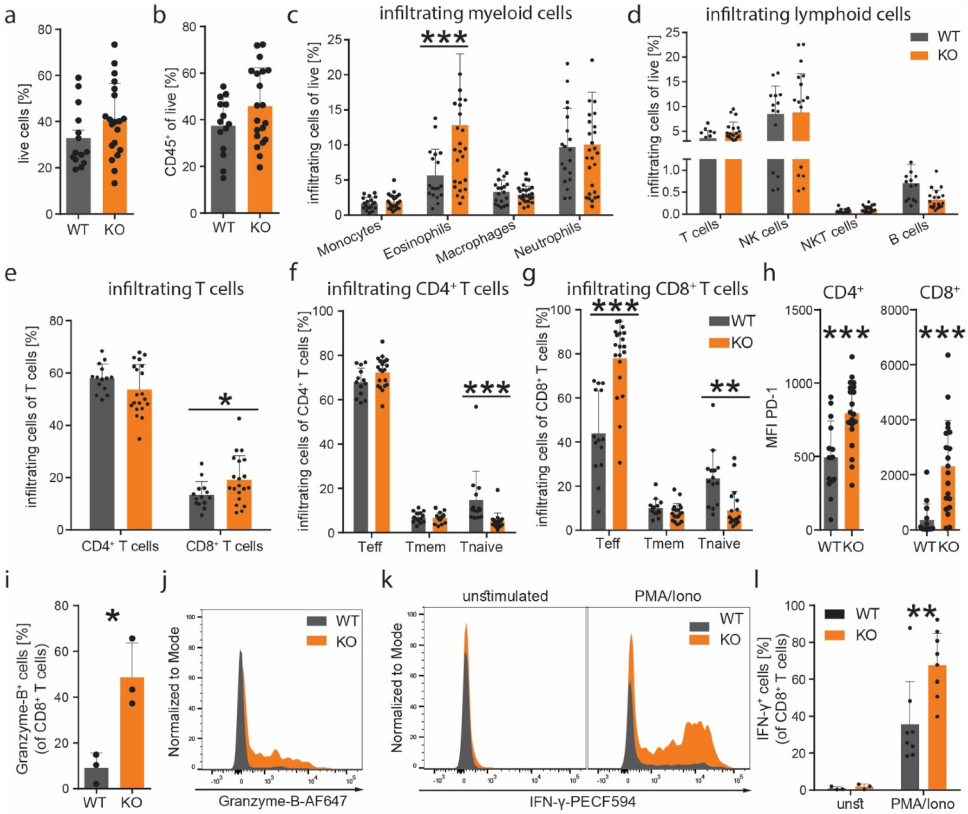

Figure 2. MGL deficiency in cells of the TME favors an anti-tumorigenic immune cell profile. (**a-g**) Flow cytometric analysis of single cell suspensions of KP cell tumors from MGL wild type (WT) and knockout (KO) mice (effector T cells, *Teff*; memory T cells, *Tmem*; naïve T cells, *Tnaive*). Data were pooled from two or three independent experiments; n = 14–20; mean values + SD. (**h**) Median fluorescence intensity (MFI) of PD-1 on CD4 and CD8$^+$ T cells is shown. Data were pooled from two independent experiments; n = 14–20; mean values + SD. (**i**) The graph shows flow cytometric analysis of granzyme-B expression of tumor infiltrating CD8$^+$ T cells from MGL WT and KO mice as well as a (**J**) representative histogram. Data indicate means + SD. n = 3. (**k**) Representative histograms of IFN-γ expression before (unstimulated, *unst*) and after phorbol 12-myristate 13-acetate/ionomycin *(PMA/Iono)* stimulation of tumor infiltrating CD8$^+$ T cells. (**l**) Quantitative analysis of tumor infiltrating CD8$^+$ T cells showing increased expression of IFN-γ in the MGL KO mice after *ex vivo* PMA/Iono stimulation. Data indicate means + SD, n = 9–10. Statistical differences were assessed by using student's *t*-test or Mann-Whitney test and multiple *t*-tests. *$p < .05$; **$p < .01$; ***$p < .001$.

MGL is expressed in tumor cells and various cells of the TME

Our first results showed that MGL deficiency in cells of the TME resulted in a lower tumor burden accompanied by changes in the TME. Since previous data showed that MGL expression in TME macrophages had an influence on tumor progression,[33] we were interested, in which cells of the TME of our NSCLC model MGL were expressed.

We used ISH technique with a specific probe against MGL combined with immunofluorescence (we have tested the specificity of this probe in MGL KO mice previously[34]). In tumors of MGL WT mice, only 40% of DAPI-stained nuclei, that stained for the tumor cell marker cytokeratin, colocalized with adjacent MGL mRNA ISH signals (Figure 3a), indicating that MGL mRNA was also expressed by TME cells. Within the TME, we detected MGL mRNA in F4/80-positive macrophages (Figure 3b), fibroblast specific protein (FSP)-positive tumor-associated fibroblasts (Figure 3c), endothelial cells (Figure 3d), but, particularly, in α-SMA positive cells (Figure 3e). Of note, primary fibroblasts from NSCLC patients and dermal fibroblasts from C57BL/6 mice differentiated by TGF-β also expressed MGL mRNA in cell cultures, hence confirming our findings (see *Supplementary figures S4a and b*). CD3$^+$ T cells did not express MGL mRNA in the tumors (Figure 3f). Around 20% of cytokeratin positive cells stained for MGL mRNA (Figure 3g). As expected, cells in the TME from tumors of MGL KO mice were devoid of MGL mRNA expression and staining for MGL mRNA in these mice was, therefore, only observed in tumor cells (see *Supplementary figures S4c-h*). MGL RT-qPCR in tumor samples of WT mice was higher than in KO mice (Figure 3h). The qPCR data in the MGL KO mice indicated that MGL was expressed in KP cells *in vivo* thus confirming our ISH data. KP cells kept in culture only marginally expressed MGL mRNA (*Supplementary figure S4i*), and equally, expression of ABHD6 mRNA was low (*Supplementary figure S4i*). MGL and ABHD6 mRNAs showed equally high expression in tumors of WT mice (Figure 3i). There were no differences in ABHD6 expression between MGL KO and WT mice (*Supplementary figure S4j*). At the same time, ABHD12 showed highly elevated expression in MGL WT tumors (Figure 3i) and cultured KP cells (*Supplementary figure S4i*), but no changes were seen between MGL KO and WT tumors (*Supplementary figure S4k*).

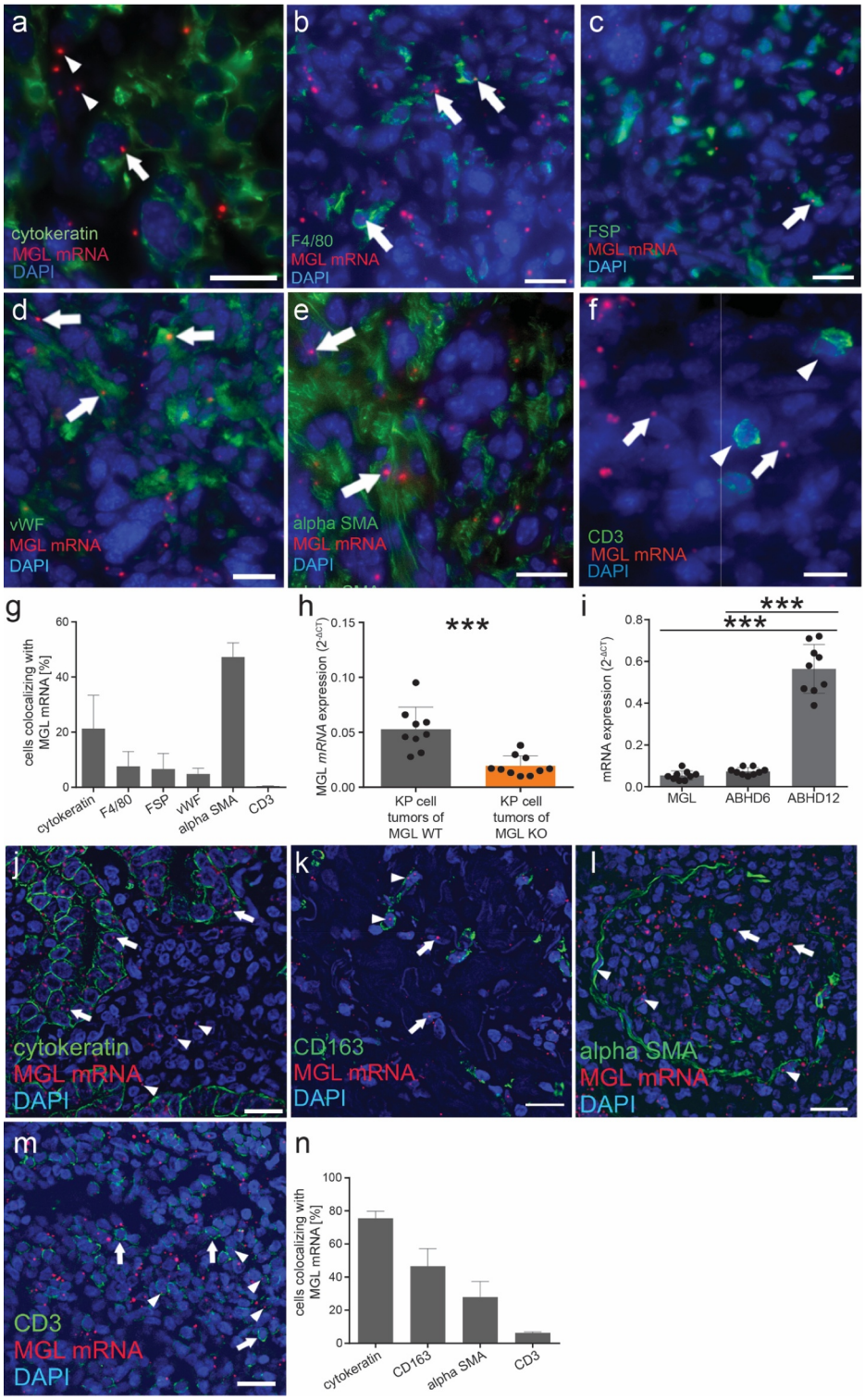

Figure 3. In situ hybridization (ISH) of MGL mRNA in KP cell tumors of MGL wild type (WT) mice. **(a)** In MGL WT mice with KP cell tumors, MGL transcripts can be seen in tumor cells (*arrow*; cytokeratin used as a tumor cell marker), and also outside cytokeratin-positive cells (*arrowheads*). MGL mRNA colocalizes with cells of the TME (*arrows*) such as with **(b)** macrophages (F4/80; ~7%), **(c)** cells expressing fibroblast specific protein (FSP; ~ 6%), **(d)** endothelial cells (von Willebrand factor – vWF; ~ 5%), and **(e)** to a large part with (alpha) α-SMA positive fibroblasts; ~ 47%. **(f)** MGL mRNA expression (*arrows*) was not observed in $CD3^+$ T cells (*arrowheads*). (**g**) Graph showing % of MGL mRNA colocalization with indicated cell populations in tumor sections. Cells from three different tumor sections were used for quantification. Between 60 and 250 cells/section were evaluated for colocalization. Data show means + SD. (**h**) Relative MGL mRNA expression in lysates of KP cell tumors from MGL WT and KO mice. n ≥ 9. Data indicate means + SD of one representative experiment. One-way ANOVA with Tukey's multiple comparison test. ***$p < .001$ (**i**) Relative mRNA expression of MGL, ABHD6 and ABHD12 in lysates of KP cell tumors from WT mice. Data show means + SD. n = 9. One-way ANOVA with Tukey's multiple comparison test. ***$p < .001$.(**j**) Similar to KP cell tumors in mice, sections of human adenocarcinoma show MGL mRNA ISH signals in tumor cells (using cytokeratin as a tumor cell marker; *arrows*) and in the TME (*arrowheads*). (**k**) MGL mRNA signals are visible in $CD163^+$ macrophages (*arrowheads*) next to other cells of the tumor (*arrows*). (**l**) Also in human lung adenocarcinoma, MGL mRNA colocalizes with (alpha) α-SMA positive fibroblasts (*arrowheads*) next to other cells of the tumor (*arrows*). (**m**) Unlike in the KP cell tumors of mice, colocalization of MGL mRNA was seen in some $CD3^+$ positive T cells (*arrowheads*). *Arrows* denote $CD3^+$ positive T cells without MGL mRNA expression. (**n**) % of MGL mRNA colocalization with indicated cell populations in tumor sections. Sections from two patients with lung adenocarcinoma were used for quantification. Between 75 and 500 cells/section were evaluated for colocalization. Data show means + SD. Calibration bars: 20 µm

In sections of human lung adenocarcinoma, most tumor cells expressed MGL mRNA (75%) (Figure 3j). In addition, we detected MGL mRNA in $CD163^+$ macrophages (46%) and in α-SMA positive cells (28%) (Figure 3 k,l). Unlike in the KP cell tumors of mice, the human samples showed MGL mRNA expression in some $CD3^+$ T-cells (6%) (Figure 3m and 3n).

In vitro, 2-AG shifts $CD8^+$ T cells toward a T effector phenotype and activates eosinophils in a CB_2 dependent manner

MGL hydrolyzes monoglycerides (MGs), and in that function it may affect 2-AG and also other lipid levels. We, therefore, measured MGs, endocannabinoids, endocannabinoid-like lipids and free fatty acids (FAs) in tumors of MGL deficient and WT mice, first focusing on changes of endocannabinoids and MGs in tumor tissues. Mass spectrometric measurements revealed increased levels of 2-AG and 2-OG in tumors of MGL KO as compared to WT mice (Figure 4a). Next to 2-AG, AEA and palmitoylethanolamide (PEA) were increased in tumors of JZL184-treated vs. vehicle-treated control mice (*Supplementary figure S5a*). When comparing 2-AG levels of KO and WT tumor tissue vs. tumor-adjacent white adipose tissue, increased levels were detected in KP tumor tissue, indicating a tumor-specific rise of 2-AG that was significantly higher in tumors of MGL KO mice (*Supplementary figure S5b*).

Second, as MGL has been reported to regulate FA levels in aggressive cancer cells,[19] we investigated whether FAs were altered in our tumor models. However, we did not detect alterations in FA contents but noted increased triglyceride levels in the MGL KO tumors instead. Many lipid species described as being altered in human lung cancer tissue, such as phosphatidylcholines (PCs,[62]) and lysophosphatidylcholines (LPCs,[63]), were found increased in tumors of MGL KO mice (*Supplementary figure S5c*).

Since we had found increased levels of 2-AG in the tumors of MGL KO vs. WT and JZL184-treated mice (Figure 4a and *Supplementary figure S5a*), we investigated whether 2-AG could play a role in the change of the infiltrating immune cell populations in MGL KO mice (specifically $CD8^+$ T cells and eosinophils) and/or tumor cell proliferation. We first investigated the role of 2-AG on differentiation, migration, and proliferation of $CD8^+$ T cells *in vitro*. Based on our finding that CD44/CD62L-expression revealed a shift to an increase in infiltrating $CD8^+$ T effector cells into tumors of MGL KO mice, as compared to WTs, we checked CD44 expression on polyclonal-stimulated T cells *in vitro*. We observed an increased CD44 expression in $CD8^+$ T cells from MGL KOs compared to WTs that was not sensitive to CB_2 inhibitor SR144528 treatment (Figure 4b). To confirm an involvement of 2-AG, we additionally treated T cells isolated from MGL WT spleens with different concentrations of 2-AG and recorded a dose-dependent increase in $CD44^+$ $CD8^+$ T cells (Figure 4c).

As a next step, we isolated T cells from MGL WT and KO mice and investigated migration in response to increasing concentrations of 2-AG (chemoattractant) in a Transwell plate. We identified dose-dependent migration of $CD8^+$ T cells toward 2-AG (Figure 4d). Since there was no significant difference in the migratory potential between MGL WT and KO $CD8^+$ T cells, we continued our experiments with WT cells. Here, the additional treatment with SR144528 showed a reduced migration compared to control (EtOH)-treated cells (Figure 4e), suggesting the involvement of a CB_2 dependent mechanism in migration.

We also investigated whether the proliferation of $CD8^+$ T cells could be affected by 2-AG. However, incubation of T cells with different concentrations of 2-AG did not change their proliferative behavior (*Supplementary figures S6a and a*). Although 2-AG was described to reduce the growth of pancreatic cancer cells *in vitro*,[64] we failed to see the effects of 2-AG on KP tumor cell proliferation and viability *in vitro* (*Supplementary figures S7a and b*).

In addition to $CD8^+$ T cells, we isolated eosinophils (which are known to express CB_2[56,65]) from bone marrow cells of WT mice and differentiated them into bone marrow-derived eosinophils (BMDEs). MGL expression was not detected in eosinophils within tumors (data not shown); therefore, the following experiments were performed in WT cells only. BMDEs were used on day 14 with purity and viability of about 93% and 94%, respectively (*Supplementary figure S8*), and we tested the chemotactic potential of 2-AG. BMDEs migrated toward 2-AG in a dose-dependent manner (Figure 4f). Migration was suppressed after pre-treatment with 1 μM of CB_2 inhibitor SR144528, corroborating previous findings[56] (Figure 4f, representative dot plots are shown in Figure 4g). We also wanted to know whether 2-AG could induce Ca^{2+} flux in murine eosinophils via CB_2, as previously described for human eosinophils.[56] Ca^{2+} flux was dose-dependently increased in BMDEs of MGL WT mice when stimulated with 2-AG (Figure 4h). Similar to the chemotactic effect of 2-AG, pre-treatment with 1 μM of SR144528 reduced Ca^{2+} signals almost to baseline values (Figure 4h-i).

Taken together, our *in vitro* data indicate that 2-AG activates eosinophils and promotes differentiation in $CD8^+$ T cells, supporting the observed *in vivo* effects.

WT mice injected with MGL-overexpressing KP cells showed increased tumor growth

In addition to cells of the TME (Figure 3b-e), MGL is expressed in KP tumor cells *in situ* (Figure 3a), but is hardly detectable in cultures of KP cells *in vitro* (*Supplementary figure S4h*). To investigate whether tumor cell-expressed MGL affects tumor growth and the TME landscape in our model, we generated KP cells overexpressing MGL, which were lentivirally transduced either with a MGL-encoding (KP_MGL) or a control plasmid (KP_ctrl). CFP (present in the control and the MGL-encoding plasmid) was used for sorting successfully transduced cells (*Supplementary figure S9a*). MGL overexpression in the transduced KP cell line was confirmed by qPCR and MGH activity assay (*Supplementary figure S9b-c*). WT mice were injected s.c. with either the MGL-overexpressing

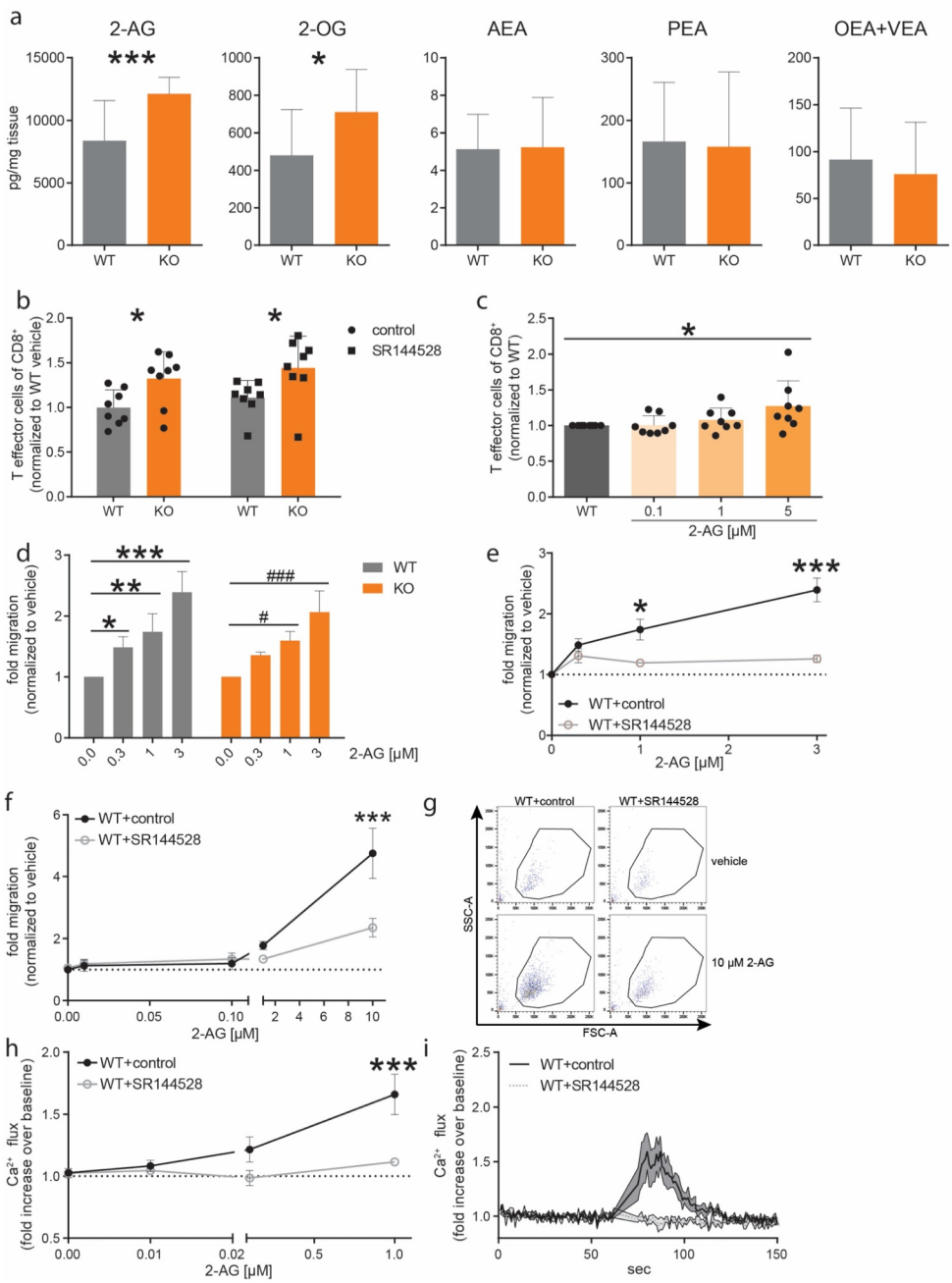

Figure 4. 2-AG shifts $CD8^+$ T cells toward a T effector phenotype and activates eosinophils *in vitro*. (**a**) Tumors were snap frozen in liquid nitrogen and the tissue homogenate was subsequently analyzed by mass spectrometry. Graphs show lipid levels of MGL WT and KO tumors (n ≥ 12). (**b**) T cells were isolated from the spleen of WT and MGL KO mice and incubated for 3 days with anti-CD3/CD28-antibodies as well as pre-treated (30 min) with 1 µM of the CB_2 antagonist SR144528 or control (<0.05% EtOH). (**c**) T cells isolated from WTs were treated with anti-CD3/CD28-antibodies and with increasing concentrations of 2-AG (or vehicle [<0.05% DMSO]). T cell differentiation was detected by flow cytometry. Data show means + SD from three independent experiments normalized to CD44 expression of WT vehicle-treated T cells. n = 8. (**d**) $CD8^+$ T cells were isolated from spleens of MGL WT and KO mice and were allowed to migrate toward different concentrations of 2-AG or vehicle in a Transwell plate and counted by flow cytometry. n = 3. (**e**) $CD8^+$ T cells of WT mice were additionally pre-treated with 1 µM of SR144528 for 10 min (or control). Statistical difference of migrated (toward 2-AG) $CD8^+$ T cells WT+control compared to WT+SR144528. (**f**) Bone marrow-derived eosinophils (BMDEs) from WT (C57BL/6J) mice were allowed to migrate toward different concentrations of 2-AG or vehicle in a Transwell plate and enumerated by flow cytometry. The BMDEs were incubated with 1 µM of SR144528 (or control). Data are shown as means ± SEM from three independent experiments. n = 3–4. (**g**) Representative dot plots of migrated BMDEs. Numbers indicate BMDEs counted for 60 s on high with flow cytometry. (**h**) BMDEs from WT mice were labeled with Fluo-3-AM and flow cytometry was used to detect changes in the Ca^{2+} flux. BMDEs were pre-treated with 1 µM SR144528 or control. Subsequently, BMDEs were stimulated with different concentrations of 2-AG or vehicle only. Results represent fold increase in Ca^{2+} flux over baseline. Data are shown as means ± SEM from three individual experiments. n = 3–4. Statistical difference of WT +control vs. 1 µM SR144528 treated BMDEs. (**i**) Time course of Ca^{2+} flux in BMDEs stimulated with 1 µM 2-AG. BMDEs were either pretreated with 1 µM SR144528 or control. After baseline measurement (60 s), 2-AG was added to induce Ca^{2+} flux. Data are shown as means ± SEM or + SD from three independent experiments. n = 3–4. Statistical differences were assessed by using two-way ANOVA with Tukey's or Sidak's post hoc test, student's *t*-test and one-way ANOVA with Dunnett's multiple comparison test. *$p < .05$; **$p < .01$, ***$p < .001$.

or the control KP cell line and tumor growth was monitored for about 14 days (Figure 5a). In general, tumors in these experiments were smaller than tumors in Figure 1 (tumors with non-transfected KP cells), which may have

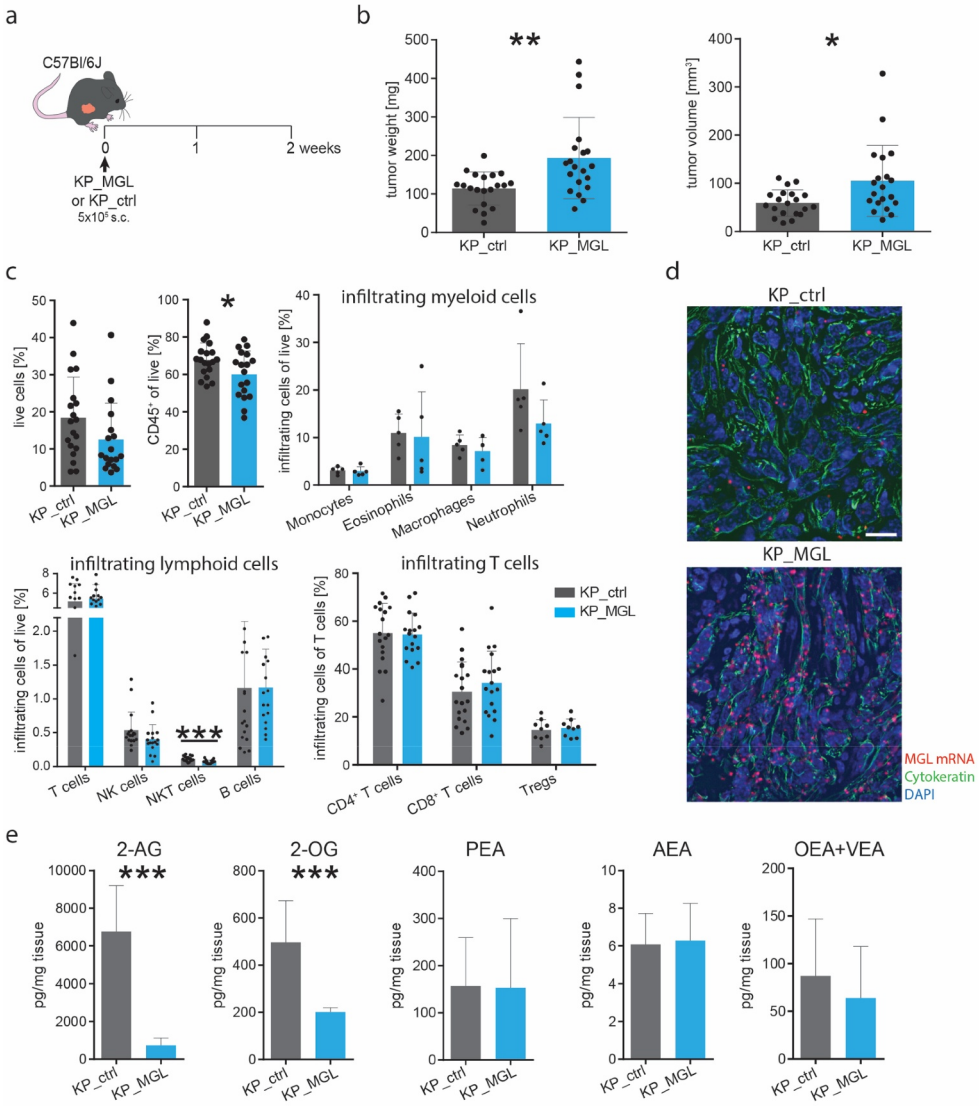

Figure 5. Increased tumor growth in WT mice injected s.c. with MGL-overexpressing KP cells. (**a**) KP cells were lentivirally transduced to overexpress MGL (KP_MGL) or a control plasmid (KP_ctrl), and afterward injected s.c. into the flank of C57BL/6J wild-type mice. (**b**) Mice were sacrificed and *ex vivo* tumor weight and volume were measured. Data indicate means + SD. Data were pooled from two independent experiments. n = 20. (**c**) Flow cytometric analysis of single cell suspensions of KP cell tumors. Data were pooled from two independent experiments; n = 18–19; for myeloid cells only one experiment is shown (n = 5). (**d**) Representative pictures of KP_MGL/ctrl tumors after in situ hybridization for MGL (MGL, *red*; cytokeratin, *green*; DAPI, *blue*). Calibration bar: 20 µm (**e**) Lipid levels in KP-MGL overexpressing (KP_MGL) and control (KP_ctrl) tumors. Data indicate means + SD. n = 8–12, pooled from two independent experiments. Statistical differences were assessed by using two-way ANOVA with Sidak's post hoc test, student's *t*-test or Mann-Whitney test and multiple *t*-tests. **p* < .05; ***p* < .01; ****p* < .001.

been due to the immunogenicity of CFP.[66] Analysis of *ex vivo* tumor weight and volume demonstrated significantly increased tumor burden in mice with the overexpressing MGL KP tumor cells (Figure 5b). Using BrdU-staining, we excluded that differences in the proliferation of the cell lines *per se* could have been responsible for the increase in tumor growth (*Supplementary figure S9d*).

Flow cytometric analysis of single-cell suspensions of tumors from MGL-overexpressing vs. control mice showed reduced infiltration of $CD45^+$ and NKT cells. However, no changes in the T and B cell profiles, nor in infiltrating myeloid cell populations (Figure 5c). The results suggest that next to TME-expressed MGL, tumor cell-expressed MGL may also promote tumor growth in our model though with minor impact on the TME immune cell profile. Increased MGL expression in tumor cells was also detected by ISH for MGL combined with immunofluorescence of cytokeratin (tumor cell marker) (Figure 5d). Endocannabinoid levels were measured with mass spectrometry and showed significantly reduced 2-AG and 2-OG levels in MGL-overexpressing KP tumors compared to control (Figure 5e). Lipid screening revealed differences in various lipid species between KP_MGL and KP_ctrl tumors (*Supplementary figure S10*). The lipid profile of MGL overexpressing tumors was less altered as compared to tumors from MGL KO mice suggesting that MGL located in TME cells may have a critical influence on the composition of the tumor lipid profile and maybe on the generation of procarcinogenic precursors. No increase of FAs was seen in MGL overexpressing tumors.

Discussion

Despite a decline since 2008, lung cancer is estimated to cause more deaths than breast, prostate, colorectal, and brain cancers combined.[67] NSCLC comprises the majority of all lung cancers. Although significant advances have been achieved with new forms of treatment, such as immunotherapy, NSCLC remains challenging to treat, calling out for a better understanding of the TME's role in this type of cancer.[68]

Lately, the immune cell landscape of NSCLC has been characterized, describing reactive T cells and neutrophils as potentially important players of NSCLC carcinogenesis.[69] Since components of the endocannabinoid system are widely present in immune cells (rev. in[32]), we wanted to address the question whether the 2-AG degrading enzyme MGL located in the TME contributes to NSCLC carcinogenesis. A link between endocannabinoid degradation and NSCLC has previously been reported in an immunocompromised mouse model demonstrating a reduction in tumor growth and metastasis by FAAH inhibitors.[70,71] Furthermore, decreased invasion and metastasis of lung cancer cells were detected upon MGL inhibitor treatment in athymic mice.[22] Other than these reports, only cannabinoids and their receptors have been studied in lung cancer cells and in *in vivo* lung cancer models (e.g.[72–74]). A study by Xiang et al. has focused on MGL inside the TME and investigated the role of MGL in macrophages and its impact on tumor growth in gastrointestinal cancer mouse models showing a significant impact of MGL on cancer progression.[33] Data are otherwise contradictory whether MGL promotes tumor growth or acts as a tumor suppressor.

The MGL/2-AG axis in the TME affects lung cancer

We applied a cell line generated from a mouse lung adenocarcinoma in a syngeneic NSCLC tumor model and studied tumor growth, MGL expression, and alterations in immune cell populations of the TME (summarized in Figure 6).

We could show that tumors with a MGL-deficient TME were smaller than tumors with a MGL positive TME. We also observed higher levels of 2-AG in MGL KO vs. WT mice tumors, most likely because of an absence of MGL expression in the TME of the KOs. Higher levels in tumors vs. normal tissue have been frequently observed for 2-AG[46] (also reviewed in[32]), and confirmed in our study. Furthermore, Qiu et al. have shown an anti-tumorigenic effect of 2-AG on tumor progression in a model of pancreatic cancer,[64] suggesting that the MGL/2-AG axis indeed could be critical in the pathogenesis of cancer. Our data also showed that MGL mRNA expression levels positively correlated with tumor burden in the NSCLC model (Figure 1h). In line with that, low expression of MGL was associated with increased survival in hepatocellular carcinoma.[75] Unlike other findings,[61] no evidence for a correlation of ABHD6 with tumor size was detected in our study, whereas the high expression of ABHD12 suggests that this enzyme could be a monoglyceride hydrolase in tumor cells next to MGL with a potential role in NSCLC pathogenesis.

Concerning MGL mRNA expression in tumor cells *in vitro*, we observed very low expression in the cultured KP cells while MGL mRNA was visibly expressed in tumor cells *in situ*, suggesting that MGL RNA expression is upregulated once KP tumor cells grow inside the body. Thus, *in situ* tumor cells as well as TME cells, many of them α-SMA-positive fibroblasts, were found to express MGL indicating that degradation of 2-AG and potential use of AA for the synthesis of other mediators like prostaglandins[10,11] could occur in cancer as well as in TME cells. We corroborated MGL in tumor and TME cells in sections of human lung adenocarcinoma by ISH/immunofluorescence, and in lung fibroblasts from NSCLC patients *in vitro*.

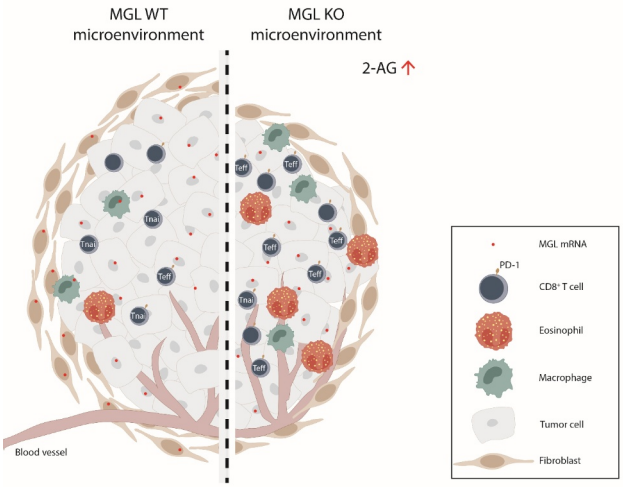

Figure 6. Deficiency of MGL in the tumor microenvironment (TME) alters the immune cell infiltrate in tumors of a non-small cell lung cancer model. Summary of differences observed in the TME cell landscape of MGL wild type (WT) and MGL knockout (KO) mouse tumors. An increase in levels of 2-AG in tumors from MGL KO mice is accompanied by increased infiltration of eosinophils and $CD8^+$ T cells. Furthermore, the number of effector cells (Teff) and the expression of PD-1^+ on $CD8^+$ T cells are increased. The altered TME cell profile in the MGL KO mice may favor tumor reduction. (Tnai= naive T cells).

MGL and 2-AG affect differentiation and tumoricidal activity of $CD8^+$ T cells

The deficiency of MGL in cells of the TME had a significant impact on the immune cell profile, which was shifted to a more anti-tumorigenic profile. Significant increases were observed for cytotoxic $CD8^+$ T cells and eosinophils in MGL KO vs. WT mice tumors. A more detailed investigation of $CD8^+$ T cells revealed that they were also more active (higher levels of granzyme B and IFN-γ) and that 2-AG may have direct effects on the conversion of T naïve into T effector cells. This is of high interest, as reduced T effector cells[76] and a potential dysregulation of T lymphocytes with reduced IFN-γ expression were detected in NSCLC patients.[77] Thus, the impact on the $CD8^+$ T cell population may represent an important mechanism against tumorigenesis generated by MGL deficiency in the TME. 2-AG has already been described to induce changes in the immune cell composition of the TME, leading to an increase in myeloid-derived suppressor cells (MDSCs) in a model of pancreatic cancer.[64] In contrast to our findings, $CD4^+$ and $CD8^+$ T cells were not affected in this model.[64] We were able to confirm a role

of 2-AG in $CD8^+$ T cell migration *in vitro*, suggesting that 2-AG may have contributed to the higher number of $CD8^+$ T cells in KP tumors of MGL KO mice *in vivo*.

We also observed increased PD-1 expression on $CD4^+$ and $CD8^+$ T cells in our study, which could be a consequence of high T cell activity against antigens in tumors of MGL KO mice.[78] When comparing PD-1^+ and PD-1^- $CD8^+$ T cells in an adoptive T-cell therapy study, authors found that only PD-1^+ $CD8^+$ T cells could control tumor progression, and that anti-PD-L1 therapy further enhanced this effect[79] adding translational value to our findings.

Collectively, our data on the immune cell infiltrate in our NSCLC model indicate that MGL derived from TME cells may suppress infiltration, differentiation and activity of $CD8^+$ T cells during tumor progression in NSCLC. MGL in TME cells is, therefore, likely responsible for maintaining a tumor progressive environment.

Direct effects of 2-AG on immune cells

Since increased 2-AG levels were measured in tumors of MGL KO and JZL184-treated mice, we hypothesized a direct role of 2-AG signaling in tumor development. As CB_1 receptors are desensitized in MGL KOs or mice chronically treated with a high dosage of JZL184,[7,80] we excluded the involvement of CB_1 in our experiments. We, therefore, concentrated our *in vitro* experiments on CB_2 and observed CB_2 dependent effects in the differentiation of $CD8^+$ T cell phenotypes and activation of eosinophils by 2-AG. However, we cannot exclude that 2-AG may have also acted via leukotriene receptors by rapid hydrolysis and leukotriene biosynthesis as previously shown.[50] Human leukocytes are known to contain several hydrolases other than MGL and could hydrolyze 2-AG.[81] In contrast to previous findings of an anti-proliferative effect on e.g. pancreatic cancer cells by 2-AG,[64] we failed to see a direct influence of 2-AG on tumor cell proliferation and apoptosis *in vitro*. As to eosinophils, they are known to exhibit pro-tumorigenic and many anti-tumorigenic properties dependent on the types of cancer (rev. in[82]). In human NSCLC, eosinophils are usually present at low levels (0.3% of all immune cells),[83] however, we counted higher numbers of eosinophils in the MGL KOs of our model (around 12%), which could have contributed to an anticarcinogenic effect.

MGL overexpressed in KP cells contributes to tumor growth

As described above, MGL is expressed in KP tumors *in vivo*, but it is hardly present in KP cells *in vitro*. To identify if tumor cell-derived MGL might contribute to changes in tumor growth and in the TME profile, we used lentiviral transduction to create a MGL-overexpressing KP cell line. Tumor growth was increased in MGL-overexpressing KP cells *in vivo*. Our data are in line with a study by Nomura et al., who showed increased MGL expression in aggressive cancer cells, and enhanced tumorigenic behavior when MGL was overexpressed in non-aggressive cancer cells.[19] Thus, together with another study showing that overexpression of MGL in nasopharyngeal carcinoma cells increased their metastatic potential,[84] MGL in KP tumor cells (next to TME-derived MGL) also governs tumor progression. However, since we failed to detect changes in the number of T cells and eosinophils (though we noted a decrease in NKT cells) in the immune cell infiltrate of the TME of MGL overexpressing tumors, tumor cell-derived MGL may have less importance than TME-derived MGL in regulating the TME immune landscape.

Lipids display distinct profiles in tumors of MGL WTs vs. KOs, and in mice with MGL overexpressing tumor cells

Many lipid species evaluated in our study were increased in MGL KO mice vs. wild-types, such as species of LPC, PCs and sphingomyelins. Some of these lipids have been found decreased in lung cancer, for instance PC (18:4/3:0) and LPC (18:0,18:1,18:2).[62,63,85] However, at the moment, we can only speculate whether an increase in these lipids could have contributed to the reduced tumor growth in the MGL KO mice. No differences in the content of FAs were observed between MGL overexpressing and normal tumors suggesting that MGL overexpressed in tumor cells may not provide FAs for procarcinogenic lipid mediators in our model.

In conclusion, we could show that MGL is present in cells of the TME in a model of NSCLC and that it impacts the tumor growth and the landscape of the TME. Identifying major mechanisms for a favorable response to immune checkpoint therapy,[86] such as increased $CD8^+$ T cell infiltration and IFN-γ production, ablation of MGL in the TME may highlight a new strategy for potential adjuvant therapy. Together with the fact that a MGL inhibitor reduced tumor growth in NSCLC suggests that MGL could be an interesting target in NSCLC anti-tumor therapy.

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Author contributions

MK, CH, JK and RS designed the experiments and supervised the study. MK, CH, KM, AS, UT, PVC, SR, DR, LB and TB participated in all the *in vivo* and *in vitro* experiments, collected data, and analyzed the results. MK and OK did the statistical evaluation and the correlations. UT performed the MGH assays, TB the ISH and immunofluorescence experiments. LB provided the human samples. LH, RG, and DT were responsible for the mass spectrometry experiments. MK, CH, JK, GG and RS interpreted the data and provided technical support. MK, JK, and RS participated in the writing of the manuscript. All authors critically revised and commented on the manuscript.

Disclosure statement

The author(s) report no conflict of interest.

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