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ONTGENY OF INTESTINAL INTRATUMOURAL MACROPHAGES AND THEIR CONTRIBUTION IN CANCER REGULATION

IRENE SONCIN
SCHOOL OF BIOLOGICAL SCIENCES
2018
ONTogeny of Intestinal IntraTumoural macrophages and their contribution in cancer regulation

IRENE SONCIN

School of Biological Sciences

A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2018
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Il mio più grande grazie va alla mia famiglia: mamma, papà, Chiara, nonna, zia Mara, Andrea e a tutti i parenti. Grazie per l'Amore incondizionato e i valori che mi avete trasmesso, e continueate a trasmettere, con il vostro esempio.

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<th>Full Form</th>
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<tr>
<td>-/-</td>
<td>Knock-out</td>
</tr>
<tr>
<td>ANGPT</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APO</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ARG</td>
<td>Arginase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CAC</td>
<td>Colitis-associated colorectal cancer</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cMoP</td>
<td>Common monocyte progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CV</td>
<td>Conventionally-housed</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Chemokine (C-X3-C motif) ligand 1</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3C chemokine receptor 1</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially express genes</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>Diphtheria toxin receptor</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGA</td>
<td>Estimated gestational age</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMP</td>
<td>Erythromyeloid progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FL</td>
<td>Fetal liver</td>
</tr>
<tr>
<td>FLT3</td>
<td>Fms like tyrosine kinase 3</td>
</tr>
<tr>
<td>Fr</td>
<td>Fraction</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>gbw</td>
<td>Gram body weight</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GF</td>
<td>Germ free</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte-monocyte precursor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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SUMMARY

Macrophages comprise a heterogeneous family of tissue-resident myeloid cells that not only contribute to the tissue homeostasis and host defence, but also can be involved in the initiation and progression of several diseases, including cancer. In fact, tumour-associated macrophages (TAMs) are a major component of the leukocyte infiltrate in several cancers showing either tumour-supportive or inhibitory phenotype, depending on the disease stage and the tissue involved. Therapeutic strategies targeting TAMs are currently under preclinical and clinical assessment, and will possibly complement traditional cancer therapies to ameliorate the disease outcomes. Therefore, a better understanding of their ontogeny and intratumoural survival mechanisms is crucial for the development of effective macrophage-based immunotherapies. While there are some evidences for breast cancer, glioma and pancreatic cancer, the turnover and self-renewal properties of TAMs in colorectal cancer have not been investigated thus far.

It is well established that in the healthy colon lamina propria (LP) tissue-resident macrophages are constantly refilled by CCR2+ circulating monocytes in order to preserve their cell pool. In this work, in addition to confirming the monocyte-dependence of LP-resident F4/80hiMHCIIhi macrophages, we have identified a previously overlooked CCR2-independent F4/80hiMHCIIlo macrophage subset, which gradually declines after birth and is almost entirely outcompeted by CCR2-dependent F4/80hiMHCIIhi macrophages in the adulthood. Interestingly, in colon adenomas of ApcMin+ mice F4/80hiMHCIIlo macrophages are not only preserved but become the dominant TAMs during tumour progression. In contrast to their LP counterparts, both MHCIIlo and MHCIIhi macrophage fractions are able to maintain their numbers mostly independent from a bone marrow/monocyte input. Reduced levels of CCR2 expression, upregulation of various cell cycle genes as well as an intratumoural milieu enriched in CSF1 facilitate their self-renewal within the tumour microenvironment. Since the depletion of F4/80hi TAMs via blocking CSF1R-CSF1 axis diminishes the tumour burden in the colon, our data underline the potential of this tumour-promoting intestinal macrophage fraction as an attractive target for successful cancer immunotherapies.
1 INTRODUCTION

1.1 Tissue-resident macrophages

Tissue-resident macrophages are a heterogeneous family of phagocytic immune cells that are involved in several physio-pathological events, including development, tissue homeostasis, host defence from infections and resolution of inflammation (REF. 1-5).

In 1908, the term “macrophages” – which means “big eaters” in Greek – was chosen by I. Metchnikoff to describe these pleiotropic professional phagocytes (6), capable of internalizing and destroying large particles such as pathogens, debris, malignant and apoptotic cells (7,8). Tissue-resident macrophages are strategically located throughout the human body. Based on their anatomical location macrophages can be subdivided into distinct specialized cell subpopulations showing clear functional differences (Figure 1.1) (9).

Figure 1.1: Distribution of macrophages. Tissue-resident macrophages can be classified into distinct types according to their location, which dictates their functional specialization. In black: name of the macrophage type; in light grey: name of the organ/tissue. Taken and adapted from Murray P.J. and Wynn T.A. Nature Review. 11, 723-37 (2011).

For example, brain microglia play a crucial role in tissue remodelling and in clearing cellular debris and dead neurons from the central nervous system (10). Osteoclasts are responsible for the bone resorption by degrading the bone matrix and for preserving bone homeostasis (11), while in the lungs alveolar macrophages constantly eliminate inhaled particles, such as allergens and dust, and microorganisms. Kupffer cells, located beside the sinusoidal vessels in the liver, are specialized in removing microbial particles and other harmful agents from the venous blood system. In the intestine, macrophages are pivotal in the recognition and clearance of enteric pathogens as well as in the development of a tolerance to food allergens and the intestinal microbiota (9). Splenic marginal zone macrophages eliminate senescent erythrocytes and suppress
innate and adaptive immunity to apoptotic cells (12). In the lymph nodes subcapsular sinusoidal and medullary macrophages recognize viruses and present viral antigens to B cells to mount an antiviral humoral response (13,14). Cardiac macrophages phagocytose dying cardiomyocytes and debris, produce pro-angiogenic and protective factors (15). Bone-marrow (BM) resident macrophages stimulate niche cells to produce and release haematopoietic stem cell (HSC) retention factors (16).

In mice, tissue-macrophages have been identified by their expression of phenotypic markers F4/80, CD11b and Fc receptors (8,9). However, recent genome-wide transcriptional profiling has revealed additional generic markers that enable to identify macrophages in different tissues (17) through immunofluorescence microscopy and multi-parameter flow cytometry (Table 1). In particular, macrophages can unequivocally be distinguished from dendritic cells (DCs) by their high expression of high-affinity IgG receptor CD64 and Mer Tyrosine Kinase (MerTK) (18).

<table>
<thead>
<tr>
<th>Macrophage subset</th>
<th>Specific surface markers</th>
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<tbody>
<tr>
<td>Alveolar macrophages (lungs)</td>
<td>F4/80⁺, SiglecF⁻, CD64⁻, MerTK⁻, CD11c⁻, CD169⁺, SIRPα⁺, CD11b⁺, CX3CR1⁻, CD206⁺, CD163⁻</td>
</tr>
<tr>
<td>Red pulp macrophages (spleen)</td>
<td>CD64⁺, F4/80⁺, MerTK⁺, MHCII⁺, CD11b⁻, CD11c⁻, Clec4F⁺, Tim4⁺, SIRPα⁺, CX3CR1⁺, VCAM1⁺</td>
</tr>
<tr>
<td>Marginal zone macrophages (spleen)</td>
<td>SIGN-R1⁺, MARCO⁻</td>
</tr>
<tr>
<td>Kupffer cells (liver)</td>
<td>F4/80, CD169, CLEC4F</td>
</tr>
<tr>
<td>Microglia (brain)</td>
<td>CX3CR1, CD45int, FCRLS, SiglecH</td>
</tr>
<tr>
<td>Langerhans cells (epidermis)</td>
<td>F4/80⁺, CD64, MerTK⁺, CD11c⁺, SIRPα⁺, CD11b⁺, MHCII⁻, EpCam⁺, CD207⁺, CD24⁻</td>
</tr>
<tr>
<td>Lamina Propria macrophages (intestine)</td>
<td>F4/80⁺, MHCII⁺, CD64⁺, MerTK⁻, CD11c⁻, SIRPα⁺, CD11b⁺, CX3CR1hi, CD68⁺, CD206⁺, Tim4⁻, αβ⁻, CD163⁻</td>
</tr>
<tr>
<td>Bone marrow macrophages</td>
<td>VCAM1⁻, CD169⁺</td>
</tr>
<tr>
<td>Subcapsular sinus macrophages (lymph nodes)</td>
<td>F4/80⁺, CD169hi, CD11b⁻, CD11c⁻</td>
</tr>
<tr>
<td>Cardiac macrophages (heart)</td>
<td>F4/80⁺, CX3CR1⁺, MHCII⁺,CCR2⁺, CD11b⁺, CD11c⁺, CD68⁻</td>
</tr>
</tbody>
</table>

Table 1: Identification of macrophage populations through a combination of surface markers. The expression levels of generic surface markers can be used to identifying tissue-specific macrophage subsets. Combining multiple markers allows a more specific characterization. (Abbreviations: -: not expressed; +: expressed; hi: high expression; int: intermediate expression; lo: low expression). Taken and modified from Mowat A.M, Scott C.L & Bain C.C. Nat. Med. 23 (11), 1258-1270 (2017).

Since phenotypic markers may not be sufficiently helpful to distinguish all macrophage subpopulations within different tissues, it is preferable to combine the phenotypic characterization with a functional analysis (19). Macrophages have been shown to be
plastic cells that shape their functions in response to external stimuli and may contribute to the initiation and progression of diseases, such as autoimmune diseases and cancer. Until recently, the classification of macrophage phenotype mostly based on in vitro experiments divides macrophages into at least two types. According to the M1/M2 dichotomy, M1 or “classically activated” macrophages contribute to the host defence by releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS) to facilitate pathogen eradication (20), and have roles in anti-tumour immunity. M2 or “alternatively activated” macrophages regulate wound healing, sustain immune-suppressive responses and play anti-inflammatory roles. The M1 activation state is induced by endotoxin, interferon (IFN)-γ and/or interleukin (IL)-1α, while M2 state is induced by IL-4, IL-10, IL-13, transforming growth factor (TGF)-β and glucocorticoids (21). M1 and M2 macrophages are believed to be the extremes of a broad spectrum of activated phenotypes (19,22). In healthy tissues, macrophages often have a mixed M1/M2 phenotype that is determined by the composition of local signals (23-25). During acute inflammation, M1 macrophages are at the forefront with their ability in killing phagocytosed pathogens and upregulating proinflammatory cytokines and ROS. At a later stage, M2 macrophages may contribute in resolving the inflammation and promoting tissue repair and angiogenesis.

A large body of evidence has suggested that macrophages are able to reversibly switch their functional phenotype depending on the local milieu of cytokines and signals. In light of this, macrophages have become attractive therapeutic targets to be depleted and/or reverted in function when their effects are detrimental or to be boosted when advantageous. Because of the challenge to selectively target an extremely diverse and plastic immune cell population, it is crucial to accurately define the source of macrophages and the mechanisms that ensure their homeostasis and activity in specific tissues in health and disease.

1.2 Tissue-resident macrophages under steady state conditions

1.2.1 Ontogeny

According to the long-held ontogeny paradigm, it was assumed that resident macrophages were continually replenished by circulating blood monocytes (26). In stark contrast, recent lineage tracing experiments have drastically revised this dogma. The current paradigm holds that the majority of tissue-resident macrophages seed the tissues of the embryo during distinct waves of embryonic haematopoiesis (27). After birth, through adulthood, these embryonically derived macrophages are able to self-maintain and preserve their population size with no or minimal contribution of
haematopoietic precursors from the adult BM (28-30), with a few exceptions later described. The embryonic haematopoiesis occurs in three sequential but overlapping waves that arise both from extra- and intra-embryonic sites (31) and each wave has the potential to give rise to fetal macrophages (Figure 1.2).

The first wave, called primitive haematopoiesis, occurs in the blood islands of the yolk sac (YS) at embryonic day 7.0 (E7.0) and gives rise to primitive precursors, named early erythromyeloid precursors (EMPs), that can generate YS macrophages without any monocytic intermediate. Early EMP-derived YS macrophages spread into the tissue of the embryo as soon as the circulatory system is completely established (from E8.5 to E10.0) (32,33). In the second wave, called transient definitive haematopoiesis, EMPs are generated in the YS haemogenic endothelium. The second wave can be further subdivided into two phases: the first phase at E7.5 generates early EMPs and the second phase at E8.25 generates late EMPs, and give rise to YS macrophages locally or migrate to the fetal liver (FL) through the blood circulation and differentiate into multiple lineages, including FL monocytes (34,35). It remains unclear whether early
and late EMPs arise from distinct progenitor cell types, or rather consist of a single population that exists along a continuum of maturation stages. The third wave, called definitive haematopoiesis, gives rise to haematopoietic stem cells (HSCs) at E8.5 in the para-aortic splanchnopleura (P-Sp) region and fetal HSCs at E10.5 from aortagonads-mesonephros (AGM) regions arising from P-Sp (34). Subsequently, both YS-derived and AGM-derived HSCs colonize the FL, which becomes from E12.5 the main haematopoietic organ within the embryo with a peak of haematopoiesis at E16.5 (35,36). According to two different models of macrophage ontogeny (32,37,40), late EMP-derived FL monocytes and/or HSC-derived FL monocytes colonize all fetal tissues, except the brain (38-40), and differentiate into tissue-resident macrophages that outcompete early EMP-derived YS macrophages (33). Fetal spleen and BM are the ultimate organs to be colonized by HSCs via the circulatory blood system. After birth, as postnatal BM haematopoiesis begins, FL haematopoiesis declines and the BM-HSCs and splenic HSCs become the definitive source of circulating monocytes (41) that eventually give rise to tissue-resident macrophages in certain tissues.

Notably, human and mouse embryonic haematopoiesis systems are similar (Figure 1.3). In humans, the first wave occurs in the YS blood islands at day 18-19 of estimated gestational age (EGA), while the generation of HSCs at week 5-7 of EGA. The haematopoiesis moves to the fetal liver until week 22 of EGA and the BM haematopoiesis is established at about week 10.5 of EGA (27).

![Figure 1.3: Macrophage ontogeny in human tissues.](image-url)

Macrophages present in adult tissues derive from at least three distinct sources, such as early EMP, late EMPs and HSCs. During primitive haematopoiesis, YS-derived early EMPs give rise to the brain microglia. During definitive haematopoiesis, YS-derived late-EMPs seed the fetal liver to become the major source of tissue-resident macrophages in the lung, liver, kidney (shown in figure), pancreas, and spleen. Fetal HSCs seed the fetal BM and can potentially give rise to tissue-resident macrophages, such as intestinal macrophages. (Abbreviations: EMP: erythromyeloid progenitor; HSC: haematopoietic stem cell; YS: yolk sac; FL: fetal liver; TRM: tissue-resident macrophages). Taken and modified from Guerriero J.L. Trends Mol. Med. 24(5), 72-489 (2018).
At birth, in mice, all tissues are populated with fetal macrophages. Nevertheless, the origin of adult macrophages can vary depending on the tissue (38,39) (Figure 1.4). For instance, in the brain the adult microglia are YS-derived and persist throughout the adulthood, maintaining their seed population (40,42-44). This is likely due to the establishment of the blood-brain barrier (BBB) (45) during the embryonic development (E13.5) and the subsequent inaccessibility of the tissue to other circulating precursors. In the epidermis, FL-derived macrophages do not fully replace YS-derived macrophages; hence the Langerhans cells have a dual origin (46). In all other tissues, tissue-resident macrophages are derived from FL-monocytes that are generated in the definitive haematopoiesis, and are maintained in the adulthood independently from any contribution of adult BM-derived monocytes (47-49). On the contrary, in certain tissues such as dermis (50,51), mammary gland (52,53), heart (54,55), pancreas (56) and intestine (40,57-59), tissue-resident macrophages are replenished by adult BM-derived monocytes – with different turnover rates – in order to retain their cell pool during adulthood.

Figure 1.4: Ontogeny of adult tissue-resident macrophages can vary between tissues and can be classified based on the precursors. In the illustrated classification, group I contains uniquely the microglia, which is fully YS-derived. Group II contains Langerhans cells, which are a mixture of YS-derived and FL-derived macrophages, and the subgroup of Kupffer cells, alveolar macrophages and red pulp macrophages that are FL-derived. Tissue-resident macrophage pools in group II stop to be refilled around E15.5–17.5; thereafter maintain independently their populations in steady state conditions. In group III, the recruitment of FL-derived kidney macrophages and a subpopulation of FL-derived dermal macrophages occurs mainly during the embryogenesis but continues at low level during the adulthood. Group IV clusters tissue-resident macrophages that after birth are continuously refilled by new BM-derived precursors, such as for a dermal macrophage subpopulation, colon and peritoneal macrophages. (Abbreviations: Gr: group; E: embryonic day; YS: yolk sac; AGM: aorta-gonads-
It is widely believed that anatomical sites with constant or sporadic exposure to microorganisms and their products, such as in the intestine (60), or subjected to mechanical stress, such as in the heart (55), are characterized by a low-grade chronic inflammation that favours the recruitment and differentiation of BM-derived monocytes. However, this view might oversimplify the complexity of triggers that determine the ontogeny shift, as alveolar macrophages, for example, maintain their FL-derived pool despite being continually exposed to the lung microbiota (27). According to recent evidence, the functional specialization of tissue-resident macrophages might not be “decided” by distinct developmental origins, but rather by the local environment, which seems to determine macrophage identity and function (61).

1.2.2 Macrophage maintenance by local proliferation

Macrophages have long been considered as quiescent terminally differentiated cells that are constantly refilled, particularly in those tissues exposed to the environment, by BM-derived monocytic precursors (26). However, recent evidence suggests that macrophages can vigorously proliferate under challenging conditions, for instance during embryogenesis (43,46) and infection (48,62), and preserve a low homeostatic proliferation rate in the adult tissues, under steady state conditions, to replenish their cell pool (63). Tissue-resident macrophages such as Langerhans cells, Kupffer cells, microglia, alveolar macrophages and splenic macrophages have the potential to proliferate (48-51,64-67). However, in certain organs such as the intestine, macrophages lose their ability to proliferate (59), whereas a proportion of kidney macrophages and heart macrophages is refilled by monocytes, rather than local proliferation, with ageing (38,44,68). Under infection and acute inflammatory conditions, tissue-resident macrophages are actually prone to undergo necroptosis (69, 70); however the residual macrophages are able to restore their numbers via in situ proliferation. For instance, peritoneal macrophages that survive during the acute inflammation repopulate the tissue through an intense proliferative burst (71). During skin inflammation, a consistent proportion of Langerhans cells expands by local proliferation (64). After conditional ablation of microglia in adult mice, the microglia is rapidly reconstituted, independently of BM–derived precursor cells (65). The mechanisms that enable tissue-resident macrophages to self-maintain are still under investigation. However, it is known that several cytokines can trigger macrophage proliferation as well as the inactivation of mechanisms involved in cell cycle withdrawal (71,72). Importantly, this does not induce any neoplastic transformation or alteration in
the functional phenotype (73). Depending on the tissue, cytokines such as macrophage-colony stimulating factor (M-CSF or CSF1), IL-34, IL-4 and granulocyte macrophage-CSF (GM-CSF or CSF2) can contribute to macrophage self-renewal. As an example, CSF1R-CSF1 signalling can trigger Ras and Erk signal transducers to activate Ets1,2 transcription factors, which ultimately lead to the transcription of cyclins D and Myc (73,74). Myc can induce cyclins D as well as p53, suggesting a cross-antagonistic mechanism to promote self-renewal without causing tumorigenic transformation (75).

These recent findings, which have changed the long-held view regarding the maintenance of mature differentiated cells, have now raised numerous unanswered questions on the self-renewing potential such as:

a) Within a certain tissue, are all macrophages equally able to self-renew?

b) May distinct macrophage subpopulations have different survival and proliferation rates?

c) If so, are these properties inherited from developmental programmes or defined by local niche-specific stimuli?

Moreover, it is still debated whether the self-maintenance may rely on a local niche of progenitor cells or whether it exclusively depends on the ability of macrophages to undergo mitosis. A combination of both scenarios cannot be excluded.

1.3 Intestinal resident macrophages

1.3.1 Anatomy and immunity of the colon

To better understand the ontogeny of tissue-resident macrophages in the large intestine (or colon), a brief overview on its anatomical architecture and mucosal immune system is hereafter described. The colon, similarly to all sections of the gastrointestinal tract, consists of four layers, i.e. mucosa (from the lumen: epithelium, lamina propria (LP), and muscularis mucosae); submucosa; muscularis externa (circular muscle layer and longitudinal muscle layer); serosa (76) (Figure 1.5).

![Figure 1.5: Four-layered architecture of the colonic wall. Taken and adapted from Encyclopedia Britannica (2013).](image-url)
The colonic epithelium consists of crypts that are composed by three types of epithelial cells, namely adsorptive enterocytes, entero-endocrine cells and goblet cells. Adsorptive enterocytes and entero-endocrine cells are responsible for the digestion and absorption of ingested dietary constituents. Goblet cells produce large amounts of mucins, which are the major component of the mucus layer covering the epithelium \( (77) \). Epithelial stem cells are usually located in the lower part of the crypt, whereas the differentiated cells migrate to the apical part to constitute the epithelial surface \( (78) \). Within the epithelium, DCs and intraepithelial lymphocytes sample the antigens from the mucus layer and contribute to the gut epithelium homeostasis \( (79) \). Numerous cell populations of the immune system are located in the LP, in isolated lymphoid follicles and in the draining mesenteric lymph nodes (MLNs). Eosinophils, mast cells, B and T lymphocytes within the LP - the connective tissue underlying the epithelial layer - can modulate the intestinal microenvironment by secreting antibodies, cytokines and chemokines. On the other hand, antigen presenting cells (APCs) such as DCs initiate the adaptive immune response by processing and presenting gut antigens to naïve T cells in the draining MLNs, and by secreting various mediators involved in the regulation of the intestinal inflammation \( (80-84) \) \( (Figure 1.6) \).

**Figure 1.6: Cell composition in the colon and in the draining MLN.** The colon is constituted by an epithelial layer that forms crypts. Epithelial stem cells are usually located at the bottom of the crypts (in violet) while differentiated epithelial cells migrate to higher level (in white) migrate to a higher position. Other cell types such as Goblet cells (in light blue), intraepithelial lymphocytes and endocrine cells are embedded within the epithelial layer. The LP is infiltrated by several types of immune cells, including macrophages, dendritic cells, neutrophils, T and B cells. Isolated lymphoid follicles in the colon contain dendritic cells, B and T cells, which are crucial for antigen presentation from the colonic lumen. APCs migrate to the MLN through afferent lymphatic system and trigger the amplification of the immune response. Taken and adapted from Meng, J., et al. Front. Microbiol. 6, 643–654 (2015).
1.3.2 Functions of tissue-resident macrophages in the intestine

Macrophages are abundant in all layers of the unperturbed intestine, including the LP of the mucosa, the muscularis externa and the serosa. They are located in a unique environment, which is constantly exposed to commensal microbiota, food antigens and enteric pathogens (82). Hence macrophages, as well as the other mucosal immune cells, have evolved functional specializations to contribute to the intestinal homeostasis. In the steady state LP, resident macrophages are closely associated with the epithelial layer and are crucial immune effector cells of the innate immunity, specialized in the phagocytosis and degradation of pathogens. Moreover, they are active players in maintaining the integrity of the epithelial layer. They clear dead epithelial cells and debris, and produce prostaglandin E2 (PGE2) (85), hepatocyte growth factor (HGF) (86) and members of the Wnt signalling pathway that promote proliferation of epithelial precursors (87-89). Contrarily to dendritic cells, macrophages are sessile cells that do not migrate to the draining MLNs and display negligible activity to prime naïve T cells, which are found only in secondary lymphoid organs and not in the mucosa (90). Despite being highly phagocytic cells, mucosal macrophages are anergic to Toll-like receptor (TLR) stimulation, hence do not produce nitric oxide (NO), ROS and pro-inflammatory cytokines in response (58). They constitutively produce the anti-inflammatory cytokine IL-10 (91-93), and low levels of pro-inflammatory mediators tumour necrosis factor (TNF)-α and IL-1β (94,95). This balanced phenotype of partial activation shapes an environment of immune-surveillance and tolerance, where acute inflammation is prevented under homeostatic conditions.

In mice, mature LP macrophages are characterized by high expression levels of major histocompatibility complex type II (MHCII), CD64, Fcγ receptor 1 (FcγRI) and the chemokine receptor CX3CR1, as well as receptors specialized in the phagocytosis and uptake of apoptotic cells, such as CD163, CD206, TIM4, αβ integrin and CD36 (58, 96-98). Emerging evidence suggests that intestinal macrophages are heterogeneous and can be divided into several subpopulations characterized by overlapping and distinct surface markers, functions and locations (3) (Table 2). CD11b+CX3CR1+ macrophages include both CD11c+ and CD11c- cells, yet the differential functional specialization remain unknown (99). A subpopulation of CD169+CX3CR1+ macrophages has been found closely located to the intestinal crypts (100). In the muscularis mucosae a resident CX3CR1+ macrophage subset is specialized in communicating with enteric neurons to regulate the gastrointestinal motility (101), nevertheless the environmental factors influencing their functional specialization are still to be clarified.
### Table 2: Macrophage subsets in mouse intestine.

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<th>Location</th>
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<th>Functional specialization</th>
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<tr>
<td>Lamina Propria</td>
<td>CD64&lt;sup&gt;+&lt;/sup&gt;, CX3CR1&lt;sup&gt;+&lt;/sup&gt;, CD11c&lt;sup&gt;+&lt;/sup&gt;, F4/80&lt;sup&gt;+&lt;/sup&gt;, CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>Lamina Propria</td>
<td>CD64&lt;sup&gt;+&lt;/sup&gt;, CX3CR1&lt;sup&gt;+&lt;/sup&gt;, CD11c&lt;sup&gt;+&lt;/sup&gt;, F4/80&lt;sup&gt;+&lt;/sup&gt;, CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
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<tr>
<td>Crypt proximity</td>
<td>CD64&lt;sup&gt;+&lt;/sup&gt;, CX3CR1&lt;sup&gt;+&lt;/sup&gt;, CD169&lt;sup&gt;+&lt;/sup&gt;, F4/80&lt;sup&gt;+&lt;/sup&gt;, CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>Muscularis mucosae</td>
<td>CD64&lt;sup&gt;+&lt;/sup&gt;, CX3CR1&lt;sup&gt;+&lt;/sup&gt;, F4/80&lt;sup&gt;+&lt;/sup&gt;, CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Communication with neurons</td>
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1.3.3 Ontogeny

The ontogeny of adult intestinal resident macrophages is affected by the drastic changes in the intestine that occur after birth, such as the exposure to the microbiota, microorganisms and dietary components. In fetal and newborn colon, all tissue-resident macrophages are YS- and FL-derived and actively proliferate to sustain their cell pool, similarly to other macrophage populations such as alveolar macrophages and Kupffer cells. These cells are characterized by high expression levels of F4/80 and mainly do not express MHCII (F4/80<sup>+</sup>MHCII<sup>-</sup> macrophages). However, at around 2-3 weeks of age, the proliferative ability dramatically decreases; moreover signals from commensal bacteria and other triggers influence the recruitment and differentiation of circulating monocytes (59). FL-derived macrophages are progressively replaced by macrophages that originate from circulating blood monocytes, which are attracted into the intestinal tissue and differentiate locally into mature F4/80<sup>+</sup>CX3CR1<sup>+</sup>MHCII<sup>+</sup> macrophages through a series of short-lived intermediates in a process called “monocyte waterfall” (58,102). According to the scenario illustrated in Figure 1.7, at this stage the macrophage population in the colon LP is constituted by F4/80<sup>+</sup>MHCII<sup>-</sup>YS- and FL-derived macrophages together with F4/80<sup>+</sup>MHCII<sup>-</sup> macrophage intermediates and F4/80<sup>+</sup>MHCII<sup>+</sup> macrophages of the “monocyte waterfall”. In adulthood, the colon contains only monocyte-derived F4/80<sup>+</sup>MHCII<sup>+</sup> macrophages, refilled by the “monocyte waterfall”.

Their dependency on monocyte refilling was ultimately proved in mice lacking either the chemokine receptor CCR2 or its ligand CCL2. These particular mouse strains have both markedly reduced numbers of intestinal macrophages (59), underlying the importance of the CCR2–CCL2 axis in maintaining the macrophage pool in the intestine. Currently the mechanisms of CCL2 production in the colon are still unknown. Commensal microbiota may influence the persistence of the “monocyte waterfall”, although studies done in germ free mice have shown contradicting results (59,103-105).
Introduction

Figure 1.7: Change in macrophage ontogeny in the intestine. The dynamics of macrophage maintenance in the intestine are illustrated, from the left, with three timeframes, i.e. fetal/newborn, 2-3 weeks of age, adulthood. In the fetal/newborn colon, conditioned by the local environment, all macrophages are F4/80^hi^CD11b^lo^CX3CR1^hi^CD64^+^ and mainly MHCII^−^; display high levels of in situ proliferation; secrete IL10. These cells are shown in pink. Sustained proliferation is indicated by a continuous arrow (left illustration). At 2-3 weeks old, the onset of "monocyte waterfall" is influenced by microbial signals and gives rise to F4/80^lo^CD11b^+^CX3CR1^hi^CD64^+^Ly6C^−^MHCII^−/^+^macrophages (shown in blue) that produce IL10 and are highly phagocytic. Concurrently, F4/80^hi^MHCII^−^macrophages (in pink) stop to proliferate (dotted arrow). In adult mice, all intestinal macrophages depend on the "monocyte waterfall". Embryonically derived F4/80^hi^MHCII^−^macrophages are fully replaced by monocyte-derived F4/80^lo^CD11b^+^CX3CR1^hi^CD64^+^Ly6C^−^MHCII^+^macrophages via CCR2-CCL2 axis (in blue).

A three-parameter “niche model” (106) has been recently proposed to explain the diverse ontogeny of tissue-resident macrophages, which could be extrapolated to intestinal macrophages (Figure 1.8). According to the authors, adult tissues can be characterized by distinct criteria of “accessibility” and “availability”. “Accessibility” refers to the absence of anatomical barriers. For instance, the brain is a non-accessible tissue as the BBB prevents BM-derived progenitor cells to enter. With regards to the "availability", each tissue is constituted of a restricted number of available niches that can be occupied. Available niches may result from tissue growth during the neonatal phase or from macrophage necroptosis, for example during inflammation (explained in section 1.4). Within a tissue distinct progenitor cells may compete for the available niche and only the most suitable cell is able to engraft (106). Following this model, the intestine is an accessible tissue as no anatomical barrier prevents the seeding of progenitor cells from the blood circulation. In the neonatal window (2-3 weeks after birth) a shift in the macrophage ontogeny is observed. At this age the intestine is still undergoing a process of maturation, hence the tissue growth generates newly available niches. Furthermore, a novel external environment (i.e. microbiota, dietary...
components) may alter the local niche, thus influencing the onset of different precursors competing for the engraftment in the available niches. As a result, circulating BM-derived monocytes seem to be the most competitive precursors that differentiate into macrophages, while YS- and FL-derived macrophages fail to self-maintain. In adulthood, even though the organ growth has terminated, the continuous macrophage death of monocyte-derived macrophages, which have an exceptionally short half-life of 3-5 weeks (107), induces constant niche availability. The authors have suggested that monocyte-derived macrophages are prevented to self-maintain perhaps by the same mechanism that prevents embryonically derived macrophages to self-maintain in the intestine (30,108). The available niches are then occupied exclusively by BM-derived monocytes since YS-/FL-derived macrophages have lost their capability to proliferate and have progressively disappeared (59). The mechanisms that determine the failure to self-renew remain poorly understood.

Figure 1.8: Three-parameter “niche model” for determining the origin of tissue-resident macrophages. From the top, the first aspect to consider is the niche accessibility. If the niche is not accessible, the progenitor will not engraft. If it is, the second aspect that determines the engraftment is the niche availability. If all niches are occupied, the progenitor will not engraft. Only if a niche is available the precursor will be able to engraft and subsequently differentiate into resident macrophage. The third aspect to consider is the competition between progenitors for the niche. If there is no competition, the only progenitor will engraft. In tissues with competing progenitors, the most competitive progenitor outcompetes other progenitors and will engraft. Progenitor cell: red; most competitive progenitor cell: green). Taken from Guilliams M. & Scott C.L. Nat. Rev. Immunol. 17(7), 451-460 (2017).

1.3.4 Monocytes: reservoir for macrophage maintenance on demand

In adult mice, monocytes mainly arise from the BM and require CSF1 to develop and survive (109,110). They are continuously produced from HSCs, which differentiate through a series of cell precursors: granulocyte-monocyte progenitor (GMP),
macrophage and DC precursor (MDP), and common monocyte progenitor (cMoP). Lineage-committed cMOPs give rise to monocytes, but not DCs (111).

In mice, two monocyte subsets arise from cMOPs: Ly6C\(^{hi}\)Gr1\(^{-}\)CD43\(^{-}\)CD62L\(^{-}\)CX3CR1\(^{lo}\)CCR2\(^{-}\)VEGFR1\(^{hi}\) classical monocytes and Ly6C\(^{lo}\)Gr1\(^{-}\)CD43\(^{-}\)CD62L\(^{-}\)CX3CR1\(^{hi}\)CCR2\(^{-}\)VEGFR1\(^{lo}\) non-classical monocytes (Figure 1.9). Recent evidence suggests that, in the steady state, Ly6C\(^{lo}\) monocytes derive from Ly6C\(^{hi}\) monocytes, most probably in the circulation (47,112). Ly6C\(^{hi}\) monocytes are the bona fide monocytes - with a half-life of 20 hours – which are recruited to sites of inflammation or demand, but also in unperturbed sites such as the intestine, to give rise to mononuclear phagocytes (59,113). Ly6C\(^{lo}\) monocytes have a half-life of 5 days and are likely to be terminally differentiated blood-resident monocytes that constantly patrol the blood vessels to assess the endothelial integrity and coordinate its repair by recruiting neutrophils (114,115).

**Figure 1.9:** Schematic illustration of the mononuclear phagocyte lineage from adult BM in mice. Macrophages and DCs are tissue-resident cells. DCs (classical and plasmacytoid DCs) are generated in the BM from HSCs that differentiate into GMPs and subsequently MDPs. From MDPs, the lineage-committed CDP gives rise to pDC and preDC. BM-derived macrophages share with DCs the common progenitor MDP. MDP can also differentiate into cMOP that in turn differentiates into Ly6C\(^{hi}\) classical monocyte. Ly6C\(^{hi}\) monocytes leave the BM and in the blood circulation can differentiate into Ly6C\(^{lo}\) non-classical monocytes. On demand - for instance during an inflammatory process - classical monocytes extravasate into the inflamed tissue and differentiate into F4/80\(^{lo}\) macrophages and eventually monocyte-derived DCs. (Abbreviations: HSC: hematopoietic stem cell; GMP: granulocyte-monocyte precursor; gran: granulocyte; MDP: macrophage-dendritic cell precursor; CDP: common dendritic cell progenitor; DC: dendritic cell; PDC: plasmacytoid dendritic cells; preDC: predendritic cell; cMoP: common monocyte progenitor; mono: monocyte). Taken and modified from Varol C., Mildner A. & Jung S. Annu. Rev. Immunol. 33, 643–675 (2015).

### 1.4 Ontogeny and function of tissue-resident macrophages in inflammation

The disruption of local homeostasis, caused by conditions of infection, pathological inflammation or tissue injury, results in an extremely altered local environment. In
Guilliams M. and Scott C.L. have summarized two potential scenarios of niche refilling during inflammation that may occur in either non-accessible or accessible tissues (106). For both scenarios, tissue-resident macrophages may act as local sensors of infection and induce the recruitment of neutrophils and microbicidal circulating monocytes. Resident macrophages have been described to undergo necroptosis and decrease drastically in numbers, a phenomenon often referred to “macrophage disappearance reaction” (69,70,106). This regulated necrosis is immunogenic and contributes to the initiation of innate responses. The so-called “emergency myelopoiesis” in the BM generates abundant pools of monocytes and neutrophils beyond the requirements of an unperturbed condition and are promptly directed into the damaged site (116).

Non-accessible tissues (Figure 1.10a) become temporarily accessible. In fact, inflammation often results in leakiness of the BBB and epithelial barriers, allowing the recruitment of cells from the circulation (117). The available niches are thus repopulated by monocyte-derived macrophages that transiently coexist with the remaining original tissue-macrophage pool, which may contribute to the niche refilling by in situ proliferation (48,55,62).

In accessible tissues (Figure 1.10b), the self-maintenance of tissue-resident macrophages prevents the engraftment of monocytes under steady state conditions. However, the inflammatory process generates available inflamed niches, in which monocytes can engraft and differentiate into macrophages (106).

Of note, the contribution of monocyte-derived macrophages to refill the macrophage population may vary depending on the organ and the nature of the injury (118,119). Monocyte-derived macrophages generally display a pro-inflammatory phenotype in the early stages of the inflammatory process and depend on the constant recruitment of monocytes. Even though pro-inflammatory macrophages are beneficial during the acute inflammatory phase by clearing invading pathogens, they also generate a concomitant tissue damage caused by ROS and T_helper1 (T_h1) and T_h17 responses (120). Thus, in the resolving phase of the inflammation pro-inflammatory macrophages undergo apoptosis or may eventually switch into anti-inflammatory cells (1). It is still not clear whether activated monocytes develop into long-lived resident macrophages or whether into short-lived macrophages. It cannot be excluded that monocyte-derived macrophages may be retained in the tissue and become able to self-maintain.

In the case of the intestine, the best example of accessible tissue with available niches under steady state conditions, upon perturbation macrophages are still monocyte-derived, hence the ontogeny does not change. Inflammation-triggered monocytes and macrophages abundantly infiltrate the intestine (121) whereas tissue-resident
macrophages do not change in numbers, or are even reduced, compared to the steady state intestine (58,91,103,122). These newly recruited cells are highly TLR-responsive pro-inflammatory cells (58) that robustly secrete IL-1β, IL-6, TNFα, IL-23, NO and ROS (57,58,102,103,122,123), which facilitate the pathogen clearance. In parallel, chemokines such as CCL2, CCL3, CCL4, CCL5, CCL8 and CCL11 attract more monocytes, eosinophils, neutrophils and T cells to orchestrate the inflammatory response (124-126). Similarly to the steady state intestine, the enhanced recruitment of monocytes into the inflamed intestine is driven mostly by CCR2-CCL2 axis (121,127) although other axes, such as CCR1-CCL3 (128), might be involved. Notably, during inflammation the newly generated monocyte-derived macrophages seem to be immature cells that have not acquired the anti-inflammatory properties, such as IL-10 production and TLR hyporesponsiveness (58,103,122). It is still not clear whether local signals condition the arrest towards a full differentiation or monocytes that enter the inflamed tissue have already been committed to give rise to pro-inflammatory macrophages (106). Moreover, the fate of the remnant mature tissue-resident is yet unknown: can they also contribute to intestinal inflammation by switching their anti-inflammatory function?

During the resolution, macrophages with anti-inflammatory properties outcompete the inflammation-associated macrophages and drive the process of tissue repair as well as homeostasis restoration in the intestine (129).

Figure 1.10: Application of the “niche model” in inflammation. (a) In a non-accessible tissue, such as brain, at the inflammation onset the leakiness of the barrier allows monocytes to enter. Moreover, inflammatory stimuli activate resident macrophages, infiltrating monocytes and alter the niche itself. This event leads to macrophage death, creating niche availability, which is filled by monocytes as the remaining resident macrophages are not compatible with an inflamed
niche. As shown at the inflammation peak, monocytes in the inflamed niches may differentiate into inflammatory macrophages. Upon resolution of inflammation, the barrier permeability is restored, the niches will return to the steady state, inflammatory macrophages will undergo cell death remaining resident macrophages will be the ones repopulating the empty niches. (b) In an accessible tissue, such as liver, monocytes can enter in the steady state but do not engraft, as the niches are not available. During the resolution phase, since the tissue is not isolated by a barrier, the available niches, created by the inflammation, can be refilled both through proliferation of the remaining resident macrophages and monocyte differentiation into macrophages. Taken and adapted from Guilliams M. & Scott C.L. Nat. Rev. Immunol. 17(7), 451-460 (2017).

In conclusion, both non-accessible and accessible inflamed tissues are likely characterized by the co-existence of resident macrophages, which best adapt to the steady state niches, and monocyte-derived macrophages, which adapt to the inflammatory environment and thus unsuitable for steady state niches, and are the major source to expand the macrophage pool. Upon resolution of the inflammation, the macrophage composition is likely restored to the steady state.

1.5 Tissue-resident macrophages in cancer
1.5.1 The tumour microenvironment

A solid tumour, referred as “a wound that does not heal” (130) for its chronic inflammation, consists not only of neoplastic cells but also many other components, such as fibroblasts, mesenchymal cells, extracellular matrix (ECM), tumoural neovasculature, pericytes and several cells of the innate and adaptive immune system, including tumour-infiltrating lymphocytes (TILs), tumour-associated macrophages (TAMs), dendritic cells, eosinophils, neutrophils, monocytes and myeloid-derived suppressor cells (MDSCs). Indeed, the presence of remarkably high numbers of leukocytes in tumours was originally described by R. Virchow in 1863. This heterogeneous and intricate environment has been successively called tumour microenvironment. (Figure 1.11) (131,132).

![Figure 1.11: Schematic illustration of the cells and components that create the tumour microenvironment.](image)

Primary solid tumours consist of an intricate microenvironment comprising...
cancerous epithelial cells, endothelial cells of the tumoural vasculature, stromal fibroblasts, the ECM, secreted cytokines and growth factors, and several types of BM-derived cells, including TAMs and MDSCs. Taken and adapted from Joyce J.A. and Pollard J.W. Nat. Rev. Cancer. 9(4), 239-52 (2009).

In carcinomas, even though the main experimental focus has always been the malignant transformation of the epithelial cells, the overlooked tumour-promoting inflammation has recently been recognized as a pivotal contributor for a variety of neoplasms, and was recently added as new hallmark of cancer (133,134). This insight has thus opened a new era in the field of cancer therapy, where inflammation-related mechanisms have become a compelling therapeutic target (135,136). According to a large body of evidence the tumour microenvironment is immunosuppressive (137,138), perhaps as a result of a progressive adaptation called “immunoediting” (139). The phenotype of infiltrating immune cells is thus edited from being anti-tumoural and cytotoxic to immunosuppressive and trophic, promoting tumour progression and metastasis.

It has been shown that several solid tumours are abundantly populated with TAMs, and these cell population can contribute to the clinical prognosis (140). Both in pre-clinical and clinical studies, TAMs accumulate along with the tumour progression. Clinical studies have highlighted a correlation between abundance of TAMs and poor prognosis for breast, prostate, ovarian and cervical cancers (141). Thus far, contradictory data still persist for lung and stomach cancers (141), as well as for colorectal cancer (142-154). Overall, more than 80% of the clinical studies demonstrated a correlation between TAMs density and tumour progression. For the cases of divergent evidence, further studies will help to elucidate whether parameters such as sample number and/or different methods might have influenced the outcome. Nevertheless, it cannot be excluded that in certain circumstances a particular composition of cytokines in the tumour milieu might foster TAMs to exert anti-tumoural responses.

TAMs have been reported to promote the tumour development by taking part in several immune and non-immune processes, including angiogenesis and ECM remodeling. The angiogenesis enables the tumour to grow beyond a certain size, as nutrients and oxygen are released through the blood circulation of the newly formed vessels (155,156). TAMs are likely attracted and cluster in avascular areas of the tumour (157), characterized by lack of oxygen (i.e. hypoxia) and a peculiar composition of chemoattractants produced in response to this condition (158). Herein, hypoxia induces TAMs to upregulate the transcription of hypoxia-inducible factor-2α (HIF2α) that, in turn, induces the production of vascular endothelial growth factor (VEGF), an important angiogenic factor (159). Macrophages may also produce IL-1, which through cyclooxygenase 2 (COX2) upregulates HIF1α that leads to an increase in VEGF.
transcription (160). Moreover, TAMs actively produce inducible nitric oxide synthase (iNOS), an enzyme responsible for the high secreted amount of NO that likely results in increased vascular flow and vasodilation (161). TAMs are therefore involved both in the formation of new vessels and in their remodeling into a coherent functional network to ensure an adequate perfusion of the hypoxic areas. Furthermore, TAMs may contribute to tumour cell invasion and metastasis by releasing matrix metalloproteinases (MMPs), which degrade the ECM and the basement membrane (162,163), and a variety of growth factors that can stimulate the proliferation and motility of cancerous cells, such as fibroblast growth factor (FGF), HGF, the family of epidermal growth factors (EGFs), platelet-derived growth factor (PDGF) and TGFβs (164,165). Interestingly, clusters of macrophages have been described in proximity of the tumour vessels, where they may facilitate the extravasation of cancer cells into the bloodstream that may disseminate and form metastasis (162). Moreover, epidemiological studies have shown that chronic inflammation predisposes to various types of cancer (166-168). Macrophages can induce genetic instability and malignant transformation by secreting high amounts of ROS and RNS that have a mutagenic potential on the epithelial and surrounding cells (166).

As above-mentioned, the tumour microenvironment is composed by a multifaceted network of several cell types. This complexity is further amplified by the differential activation state for each cell population that depends on local signals, such as CSF1, CSF2, IL-10, CCL2, hypoxia, necrosis, metabolites, and their abundance (169) (Figure 1.12).

**Figure 1.12: TAM polarization is influenced by local stimuli.** The overall functional role of TAMs in angiogenesis, cytotoxicity, immune stimulation, immune suppression, chemotaxis (inner circle) is shaped by multiple signals coming from the tumour microenvironment, such as CSF1, CSF2, IL-10, CCL2, IFN-γ and hypoxia (outer circle). Taken from Ruffell B. & Coussens L.M. Cancer Cell. 27(4), 462-72 (2015).

It has always been a challenge to functionally characterize TAMs based on surface markers because macrophages rapidly evolve in relation to the environment, especially
within tumours. **Table 3** summarizes several surface and intracellular markers, as well as secreted cytokines, that have been utilized - often in combination - to distinguish the phenotype of TAMs (170).

<table>
<thead>
<tr>
<th><strong>Surface and intracellular markers</strong></th>
<th><strong>“Pro-tumour” (M2-like)</strong></th>
<th><strong>“Anti-tumour” (M1-like)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD163, CD204, CD206, PD-L1, ARG1, YM1, FIZZI, MGL2, VEGF, MMPs, CCR2</td>
<td>CD38, CD40, CD64, CD80, CD86, CD74, CD169</td>
<td></td>
</tr>
<tr>
<td>IL-1RA, IL-10, CCL17, CCL18, CCL22, CXCL12, TGFβ, IL-12^lo^, RNS, VEGFA</td>
<td>CCL2, CCL5, CXCL9, CXCL10, CXCL16, IL-1β, IL-2, IL-6, IL-8, IL-12^hi^, IL-23, IFN, ROS, NO, TNFα, iNOS</td>
<td></td>
</tr>
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</table>

**Table 3: General markers of pro-tumoural and anti-tumoural macrophages.** (Some abbreviations: VEGF: vascular endothelial growth factor iNOS: inducible nitric oxide synthase; MGL2: macrophage galactose-type C-type lectin 2; NO: nitric oxide; RNS: reactive nitrogen species; ROS: reactive oxygen species). Taken and adapted from Guerriero J.L. Trends Mol. Med. 24(5), 472–489 (2018).

Based on the M1/M2 macrophage classification, it was proposed that TAMs are predominantly polarized towards a M2-like phenotype, thus explaining their ability to promote tumour growth and vascularization. However, TAMs with a M1-like phenotype may be present in regressing tumours as well as in necrotic areas of developing tumours. Hence, even though TAMs are mainly referred as an M2-like population, tumours may contain M1-like TAMs or even, most likely, a mixture of M1- and M2-like TAMs, or TAMs with overlapping M1- and M2-like properties (52,171). Possibly TAMs are plastic cells that may display an overall pro-inflammatory phenotype in the early tumoural stage and an immunosuppressive phenotype in advanced tumours (172). In addition, not only the stage but also the cancer type and the heterogeneity within a certain cancer type may influence the functional diversity. In a certain tumour microenvironment, at least four different areas can be detected: sites of initial neoplastic cell invasion, perivascular sites, stromal sites, and hypoxic/necrotic sites. Each tumour area may educate macrophages to exert specific functions that support the necessities of that tumour region (162). For instance, areas of invasion may contain TAMs that support cancer cell motility; in stromal and perivascular areas TAMs promote metastasis, whereas in avascular and hypoxic areas they stimulate angiogenesis. Indeed, TAMs were found within dense clusters of leukocytes at areas of collapsed basement membrane, where tumour cells are thus able to escape the boundaries of a certain tissue and migrate into the stroma of surrounding healthy tissues. On the contrary, TAM clusters at sites distant from the vasculature are more specialized in angiogenesis (162).
For example, in human renal cell carcinoma 17 distinct TAM subsets were identified, including blood monocytes, BM-derived macrophages and embryo-derived resident macrophages (173). Notably, these populations expressed combinations of both “pro-tumour” (CD163, CD204, and CD206) and “anti-tumour” (CD169 and CD38) markers. In human stage I lung adenocarcinoma, 4 distinct macrophage and 2 monocyte populations were identified (174). Compared to lung tissue-resident macrophages, TAMs had increased levels in TREM2, CD81, macrophage receptor with collagenous structure (MARCO) and apolipoprotein E (APOE), CD64, CD14, and CD11c, and lower levels of CD68 and CD206.

Given the heterogeneous and dynamic nature of the tumour microenvironment, TAMs can adapt their activation state (i.e. M1- or M2-like) and undergo a metabolic reprogramming to fuel tumour growth (19,175,176) (Figure 1.13).

Figure 1.13: Tumour progression leads to the metabolic reprogramming of TAMs. At the onset of the tumour (left panel), the tumour environment is enriched of TNF, IL-1, CSF1, CCL2, DAMPs. TAMs are mostly inflammatory and characterized by a glycolytic and fatty acid metabolism, HIF-1α activation, and an inhibited oxidative phosphorylation. This results in the production of NO, ROS, IL-1β, VEGF and TNF, which in turn contribute to the genetic instability, inflammation and angiogenesis. In established tumours (right panel), an environment characterized by nutrient deprivation, lactate accumulation and Th2-derived IL-4 enrichment favors the oxidative phosphorylation rather than the glycolysis in TAMs. Immunosuppressive TAMs promote tumour growth. Taken from Biswas S.K. Immunity. 43(3), 435–449 (2015).

M1-like TAMs increase the glycolysis rate and release lactate, in parallel they reduce the oxygen consumption in order to survive within hypoxic sites (177). Furthermore, the Krebs cycle is arrested at two points, leading to the accumulation of citrate and succinate that are essential for the synthesis of pro-inflammatory regulators (including ROS, NO and prostaglandins) and for IL-1α induction, respectively (178,179). On the
other hand, M2-like TAMs have an intact Krebs cycle and preferentially use the fatty acid oxidation and oxidative phosphorylation to respectively provide carbons to the Krebs cycle and to produce energy for tissue regeneration (178,180,181). Of note, M2-like macrophages require Arginase1 (ARG1) - enzyme that converts arginine to proline - for the collagen synthesis, which is required during the resolution of inflammation (182). In conclusion, a growing body of evidence suggests that the M1/M2 classification should not be strictly used to characterize macrophages in vivo. In future, a greater effort to integrate functional, transcriptional and/or epigenetic signatures will certainly lead to a more accurate characterization (170).

Similarly to TAMs, other immune cells in the tumour microenvironment have been proposed to undergo functional changes and to regulate cancer progression. Tumour-associated neutrophils (TANs) are also key contributors of tumour angiogenesis, invasion, metastasis, and may also cause genetic instability (183). Similarly to M1/M2 dichotomy, N1- and N2-like TANs were described to be tumour-suppressive and tumour-promoting respectively, and TGF-β is a pivotal cytokine for this polarization (184).

In the tumour, also T lymphocytes are generally impaired in function. A chronically inflamed environment may lead to T cell dysfunction, for instance due to the copious ROS levels (185). With the tumour progression, the “tumour-killing” cytotoxic response may be skewed towards an immune-permissive one, characterized by T cell “exhaustion” and abundant infiltration of immunosuppressive regulatory T cells (Tregs). These latter are presumed to suppress anti-tumour immune responses by secreting IL-10, TGF-β and IL-35 (186), and by taking part in angiogenic mechanisms (187).

Apart from conditioning DCs, macrophages and granulocytes to become immunosuppressive, the tumour microenvironment fosters the recruitment and accumulation of CD11b+Gr1+ MDSCs. Currently, two main MDSC populations have been characterized: monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs) (188).

Compared with neutrophils, G-MDSCs express higher levels of CD115 and CD244 and lower levels of CXCR1 and CXCR2. They are less phagocytic but produce higher amounts of ROS, ARG1 and myeloperoxidase (189,190). M-MDSCs express varying levels of classic monocyte markers, such as F4/80, CD115 and CCR2 (191,192). They suppress CD8+ T cells predominantly via production of ARG1, iNOS, ROS, IL-10 and TGF-β (191,193,194). This MDSC subset may also include progenitors that give rise to a population of CD11bhiGr1loLy6G-F4/80MHCII+ macrophages with potent immunosuppressive properties (191,195,196).
1.5.2 Ontogeny of TAMs

Given the extensive evidence that TAMs promote tumour progression by supporting angiogenesis and invasion, and the attractive perspective of therapeutic strategies targeting TAMs in cancer patients, current studies aim to elucidate regulatory mechanisms as well as survival mechanisms of tumour-educated TAMs. To date, some recent studies - performed in murine models of mammary, brain, lung and pancreatic cancer - have shed light on the mechanism of recruitment and maintenance of TAMs. However, the developmental origins and the dynamics of maintenance and/or refilling of resident macrophages in other cancer types, including colon cancer, have not been investigated thus far. Moreover, it is still debated whether the ontogeny of different macrophage subsets determines their roles in malignant tissues. For each cancer a comprehensive correlation analysis between functional phenotype and developmental origin is thus needed.

In a mouse model of breast cancer it has been demonstrated that inflammatory monocytes are recruited in a CCR2-dependent manner at the tumour site, where they differentiate into macrophages and are able to expand their population through in situ proliferation. Inhibition of monocyte recruitment reduces the tumour burden, suggesting that CCR2-CCL2 axis might be considered as therapeutic target for the treatment of breast cancer (52). Notably, it has been shown that the predominant macrophage population does not express M2 related macrophage markers, even though it promotes the tumour growth by modulating the adaptive immune responses (52). In another study on mammary tumours, TAMs have also been described not only to derive from monocytes but also to be capable in maintaining their numbers by self-renewal (197), thus both monocyte infiltration and proliferation play an equal role in macrophage maintenance (51,197). In discrepancy with Franklin R.A., et al. (52), in this case the impairment of CCR2-CCL2 axis does not significantly affect TAM abundance and the tumour growth (197). This evidence raises the question as to whether TAMs may have unique functional specializations and phenotype depending on the cancer type, and the assumption that all TAMs are M2-like could be imprecise, or even incorrect.

Lung tumours are also highly infiltrated with macrophages and, similarly to breast cancers, they are mainly monocyte-derived (198,199). Interestingly, while in the unperturbed mammary tissue-resident macrophages are constantly replenished by monocytes, the lungs are mainly populated by embryonically derived self-renewing macrophages. Nevertheless, within tumours from lungs and mammary tissue TAMs have the same origin.
On the contrary, a study on the ontogeny of TAMs in brain malignancies shows that gliomas and brain metastases are infiltrated by two TAM populations – YS-derived resident microglia and BM-derived macrophages – that possess distinct transcriptome signatures and activation states (200).

Similarly to brain cancer, in pancreatic tumour models a mixture of YS-derived and BM-derived macrophages has been recently described (Figure 1.14). The authors have demonstrated that TAMs of different developmental origins can exert different functions. MHCII\textsuperscript{lo} YS-derived TAMs accumulate in the tumour environment via in situ proliferation and promote the tumour progression, whereas MHCII\textsuperscript{hi} are constantly refilled by inflammatory monocytes in a CCR2-dependent manner and do not seem to contribute to the tumorigenesis.

Overall, these data suggest two potential developmental routes for TAMs. They can originate from embryonically-derived and/or monocyte-derived tissue-resident macrophages that change their phenotype in response to the tumour microenvironment, or from monocytes that directly give rise to tumour-induced macrophages (201).

**Figure 1.14: In pancreatic cancer TAMs display a dual origin.** In the pancreas, under steady state conditions, tissue-resident macrophages are YS-derived (red cells). In transgenic models of pancreatic cancer, these macrophages are characterized by low expression levels of MHCII (MHCII\textsuperscript{lo} TAMs – red cells) and accumulate via in situ proliferation during the tumour progression. The tumour is also infiltrated by MHCII\textsuperscript{hi} TAMs (violet cells) that originate from Ly6C\textsuperscript{+} monocytes. The impairment of CCR2 axis, thus the consequent ablation of MHCII\textsuperscript{hi} TAMs, did not impact on the tumour growth. On the contrary, the ablation of MHCII\textsuperscript{lo} TAMs through the combination anti-CSF1R antibody + LC reduced or inhibit the progression, by containing the fibrotic process. Indeed, a dense collagenous matrix (crossed lines) characterized advanced tumours, and YS-derived MHCII\textsuperscript{lo} TAMs are proposed to sustain the tumour progression with their pro-fibrotic phenotype. (Abbreviations: PanIN: pancreatic intraepithelial neoplasia; pancreatic ductal adenocarcinoma: PDAC; LC: liposome-encapsulated clodronate). Taken from Pollard J.W. Immunity. 47(2), 217–218 (2017).
In both tumour models of brain and pancreatic cancer the authors have demonstrated that the embryo-derived TAMs were the tumour-promoting ones \(^\text{200,201}\). In brain tumour, BM-derived TAMs had higher expression of gene transcripts involved in antigen presentation and T cell costimulation, compared to embryo-derived TAMs \(^\text{200}\). Similarly, in pancreatic cancer BM-derived TAMs were more potent in antigen presentation, whereas embryo-derived TAMs were likely involved in ECM remodelling \(^\text{201}\).

All these insights further suggest that it is important to define the macrophage ontogeny and regulation in each tumour type in order to design effective strategies of immunotherapy. Nevertheless, several questions (concerning other tumour types) are still open, such as:

1) What is the contribution of macrophage populations with different developmental origin to the tumour progression?
2) Do TAMs with different origin perform different tasks?
3) Do TAMs in other cancers have the capability to self-renew via in situ proliferation?
4) Which external stimuli and intracellular pathways are responsible for the acquired self-renewal?
5) Are TAMs able to revert their phenotype regardless of the ontogeny?
6) In case of TAM-targeting therapies, could TAM proliferation represent an obstacle?

A deeper understanding on the mechanisms of TAM maintenance, obtained in cancer mouse models, will surely give insights for human cancers, in which TAM ontogeny has not been investigated thus far.

1.5.3 Monocyte recruitment axes in the tumour

Understanding the functional role of TAMs at different tumour locations is certainly one of the main focuses of current research. The crucial aspect of monocyte contribution for TAM maintenance has not been widely characterized yet. As previously described (section 1.3.4), based on phenotype and function it is commonly accepted that two subsets of monocytes leave the BM: the classical monocytes and non-classical monocytes \(^\text{41}\). Classical monocytes contribute to the inflammatory responses induced by pathogens or tissue damage \(^\text{59,113}\), whereas non-classical monocytes constantly patrol the blood vessel and eventually extravasate to facilitate the tissue repair and to reduce the inflammation \(^\text{114,115}\). In case of monocyte-derived macrophages in the tumour, it is still debated whether TAMs originate from a single
monocyte subset, from both subsets, or from a common monocyte precursor (202). For instance, in a mouse model of breast cancer it has been proposed that Ly6C\textsuperscript{lo} patrolling monocytes preferentially home to the primary tumour site, while Ly6C\textsuperscript{hi} monocytes infiltrate lung metastases (171).

Monocyte recruitment is driven by tumour-derived chemokines and chemokine receptors expressed on monocytes. Among the recruitment axes, CD62L-CD62L ligand, CX3CR1-CX3CL1, CCR2-CCL2 and VEGFR1-VEGF-A have been proposed to facilitate monocyte recruitment into different tumour microenvironments (203-205). In the case of CCR2-CCL2 axis, CCL2 is a cytokine that induces the recruitment of monocytes/macrophages to damaged tissues and tumours (206). This axis also mobilizes BM-derived monocyte precursors to the periphery (207,208), and it has been demonstrated that CCL2 may represent the major monocyte chemoattractant in the tumour (52,88,199,209,210). Since CCR2 is highly expressed in classical monocytes, they are most likely recruited to the tissues/tumour through the CCR2-CCL2 axis. Upon extravasation Ly6C\textsuperscript{hi} classical monocytes undergo a maturation process comparable to the “waterfall differentiation” that has been described for the development of intestinal macrophages under steady state (Figure 1.15) (59,211).

Specific inhibition of CCL2 with antibodies has been shown to reduce tumour growth and dissemination in different experimental models of cancer (199,212).

Ly6C\textsuperscript{hi} monocytes may also be recruited via CD62L-CD62L ligand axis. The L-selectin CD62L is a cell adhesion molecule expressed on classical monocytes but not on non-classical counterparts, and its CD62L ligand is expressed on inflamed endothelium (213), present in tumours. The interaction CD62L-CD62L ligand enables the monocytes
to adhere (214) to the endothelium and extravasate into the perivascular region of the tumour. VEGF-A - markedly abundant in hypoxic regions of the tumour - acts as a potent chemoattractant for monocytes/macrophages (215). Its receptor VEGFR1 is highly expressed only on classical monocytes (216), which are thus likely the precursors of TAMs in hypoxic regions.

Regarding the CX3CR1-CX3CL1 axis, CX3CL1 is an adhesion molecule that mediates the rapid adhesion of leukocytes in the blood stream (217). Once cleaved, the soluble form of CX3CL1 becomes a chemoattractant mostly for non-classical monocytes, since they express CX3CR1. This suggests that CX3CR1-CX3CL1 axis may recruit non-classical monocytes into the tumour perivascular region, as CX3CR1 is not express on classical monocytes (218).

Notably, the tumour microenvironment is likely characterized by distinct regions with variable concentrations of chemoattractants, and each area may employ a preferential ligand/receptor axis for monocyte recruitment. This heterogeneity could explain a possible co-existence of both monocyte subsets and/or the prevalence of one of them in certain tumour areas.

1.5.4 TAM differentiation and survival via CSF1R-CSF1 axis

The CSF1R-CSF1 axis has been found to be crucial for TAM chemotaxis, differentiation and survival. CSF1R is a receptor tyrosine kinase that undergoes oligomerization and auto-phosphorylation upon binding to its ligands CSF1 or IL-34 (219). CSF1R is expressed at low levels on HSCs (220), at higher levels on monocytes and tissue macrophages (221) and DCs (222) but also on specialized cells, such as Paneth cells (223), neural cells (224), renal proximal tubule epithelial cells and colonic epithelial cells (225). This broad expression pattern highlights the pleiotropic role of CSF1R in several biological processes, including embryonic development, adult homeostasis, innate immunity and inflammation (225).

CSF1 is a monocyte attractant as well as a macrophage survival and polarization signal that drives TAM differentiation towards an immunosuppressive, tumour-promoting ‘M2-like’ phenotype (226,227). Depending on the concentration of CSF1, CSF1R signaling may promote diverse outcomes, such as survival, proliferation, and monocyte commitment by regulating downstream intracellular pathways. For instance, low CSF1 concentrations stimulate macrophage survival due to the inhibition of total protein degradation (228,229). A dose-dependent increase of CSF1 induces protein synthesis, hence macrophage proliferation (229). CSF1R activation directly induces monocytic
cell fate in HSCs (230) and it also instructs GMPs to differentiate into macrophages (231). As a demonstration of the fundamental role of CSF1 in macrophage homeostasis, mice lacking functional CSF1 ligands are characterized by a dramatic reduction of macrophage numbers.

In the context of cancer immunology, high intratumoural CSF1 or CSF1R expression levels have been found in several tumour types and associated with poor prognosis (232-235). In the recent years, targeting TAMs by interfering with the CSF1R-CSF1 axis has become a compelling approach in cancer immunotherapy and will be discussed in the following chapter.

1.6 Macrophages in cancer therapy

1.6.1 TAM involvement in tumour responses to therapy

The implication of TAMs, and more broadly of myeloid cells, in current cancer therapies is still largely unexplored, especially in clinics. Since decades ago, cytoreductive therapies - radiotherapy and chemotherapy - have become the first-line treatment for several cancer types; however the efficacy is sometimes limited, with severe side-effects.

Recent studies in preclinical models have shed light on the complexity of the tumour microenvironment as well as the plastic role of the immune system that could influence the clinical response to the therapies. In vitro and in vivo studies have revealed that macrophages can mediate chemotherapy resistance by providing survival factors and/or activating anti-apoptotic programs in malignant cancer cells (236-238). For instance, in paclitaxel-treated mammary tumours it has been shown that TAMs can limit the efficacy of the drug by releasing lysosomal enzymes (cathepsins) that protect tumour cells from paclitaxel-induced cell death (239). As such, the efficacy of paclitaxel was enhanced by depleting TAMs through CSF1 blockade in mouse mammary tumours (236). Likewise, TAM depletion in human breast cancer xenograft in immunodeficient mice improved the efficacy of the chemotherapy (combination of cyclophosphamide, methotrexate and 5-fluorouracil) (240). Nevertheless, TAMs may also improve the treatment outcome. In a transgenic mouse model of pancreatic cancer, local low-dose ionizing radiation stimulates the accumulation of iNOS+ TAMs, which in turn contributed to normalize the tumour vasculature and promoted CD8+ T cell recruitment, thereby improving tumour control and mouse survival (241). Oxaliplatin treatment against various mouse cancer models triggers TAMs and neutrophils to produce ROS, which in turn mediate DNA damage and apoptosis (242). Additionally, TAMs are able to accumulate large amounts of nanoparticles containing the cytotoxic drug and
subsequently release them to surrounding tumour cells, inducing a delayed apoptotic effect (243).

Hence, TAMs can either reduce or amplify the magnitude of the therapy, depending on the cytotoxic agents used and the tumour model. In clinics, this dual role may contribute to the diverse response to the therapy.

Conversely, antineoplastic therapies with cytotoxic agents may induce either TAM reprogramming or depletion, improving the therapeutic outcome. For example, in a mammary tumour model it has been described that docetaxel depletes immunosuppressive M2-like TAMs and leads to the activation or expansion of M1-like monocytes/MDSCs, which in vitro are capable of enhancing the cytotoxic T cell response (244). In mice, trabectedin inhibits the growth of fibrosarcoma by depleting mononuclear phagocytes, including monocytes and TAMs (245).

In conclusion, the effectiveness of a cytotoxic therapy may depend, at least in part, on TAM-induced resistance/tumour-supportive functions and by the capability of the drug to reprogram or deplete tumour-promoting macrophages.

1.6.2 TAMs as therapeutic targets

The gained knowledge on the relationship between the lineage and function of macrophages in cancer as well as the TAM-mediated mechanisms of drug resistance may be exploited to create new opportunities of cancer therapy. Approaches under investigation include the inhibition of macrophage recruitment to the tumour and/or survival (246), and functional re-education of TAMs to an anti-tumour phenotype (247).

Hereafter, three different approaches of macrophage-based therapy will be briefly described (Figure 1.16):

1) Inhibiting monocyte/macrophage recruitment;
2) TAM depletion;
3) TAM reprogramming.

**Figure 1.16: Some of the current strategies to target TAMs for anticancer therapy.** In the blue panel: strategies to deplete or suppress TAMs in order to obtain an anti-tumour effect. In
the green panel: strategies to induce TAM reprogramming, from pro-tumour to anti-tumour phenotype. (Abbreviations: CSF1: Colony-stimulating factor 1; CSF1R: Colony-stimulating factor 1 receptor; IDO: Indoleamine 2,3-dioxygenase; IL: Interleukin; mAb: monoclonal antibody; COX2: Cyclooxygenase 2; TGFβ: Tumour growth factor β; VEGF: Vascular endothelial growth factor; TLR: Toll-like receptor; Ang2: Angiogenin 2). Taken and adapted from Guerriero J.L. Trends Mol. Med. 24(5), 472–489 (2018).

1) Inhibiting monocyte/macrophage recruitment
Since CCR2–CCL2 and CSF1R–CSF1 axes are crucial for monocyte/macrophage recruitment to tumours and TAM maintenance, these pathways are promising targets for depleting TAMs (169,226). Different approaches using monoclonal antibodies (246, 247) and small molecule inhibitors (248,249) have been demonstrated to limit TAM accumulation and to control disease progression in various mouse models of cancer, including breast, prostate, lung and liver cancer (212,250-253). It has been shown that antibody-mediated blockade of CCL2 inhibited monocyte recruitment to primary breast tumours and to metastasis in the lungs, leading to reduced tumour growth and improved survival (212,250). In different tumour models, anti-CCL2 antibody treatment improved the efficacy of the chemotherapy (254). However, in a mouse model of breast cancer the interruption of anti-CCL2 treatment caused a rapid release of monocytes from the BM into the tumours, thus accelerating the metastatic spread (250).

2) TAM depletion
The CSF1R-CSF1 axis is crucial for proliferation, differentiation and survival of mononuclear phagocytes, particularly macrophages. Inhibition of this axis, through anti-CSF1R neutralizing antibodies or small-molecule inhibitors, has been exploited to deplete TAMs in several tumour models and in patients (255-257). In a breast cancer model the absence of CSF1 in Csf1op/op mice inhibited tumour progression and metastasis (255). In another study, an anti-CSF1R blocking monoclonal antibody led to a significant reduction in TAM numbers and increased intratumoural CD8+ :CD4+ ratio in many transplantable tumour models (246). Treatment with the same antibody resulted in a beneficial response for patients with diffuse-type giant cell tumours (246). In a clinical trial the oral administration of a small-molecule CSF1R inhibitor induced tumour regression in patients with tenosynovial giant-cell tumours (258).

Nevertheless, monocyte/macrophage ablation strategies have two limitations: they systemically deplete also non-tumoural tissue-resident macrophages, hence side effects such as increased risk of infections must be taken into account when translated to clinical use. Moreover, they may be insufficient to induce tumour regression or arrest in mice (236,259,260) because of concomitant tumour-promoting mechanisms. For instance, an increase of tumour-infiltrating MDSCs has been observed upon CSF1R blockade in various preclinical tumour models (261).
In these cases, macrophage-based therapy may improve the therapeutic outcome when combined with an additional treatment (236,262-264). Currently approaches of CSF1R-CSF1 blockade, as well as CCR2-CCL2 blockade, are under evaluation as monotherapies and in combination with cytoreductive therapies or other immunotherapies, both in preclinical models and clinical trials.

3) TAM reprogramming

Another attractive option consists in modulating TAM functions, rather than depleting them. This approach may prevent the side effects of systemic phagocyte ablation. In some studies, CSF1R blockade did not deplete TAMs, rather tuned their function.

In a genetic model of glioblastoma a brain-permeable CSF1R antagonist was utilized to inhibit CSF1R. This treatment attenuated glioma progression or promoted regression of established glioma not through TAM depletion, but downregulation of some pro-tumour genes, including ARG1 and Mannose Receptor C-Type 1 (Mrc1 or CD206) (227). In a mouse model of ovarian cancer, the CSF1R inhibitor impaired the numbers of M2-like TAMs when administered at advanced tumour stages (248). In pancreatic cancer models, although CSF1R blockade reduced TAMs infiltration it also enhanced the capability of TAMs to perform antigen presentation, thus improving T cell immunity (247).

In pre-clinical model of pancreatic carcinoma an agonistic anti-CD40 antibody induced the reprogramming of TAMs towards an M1-like phenotype with antigen presenting capabilities. This led to a short-term reduction of the tumour growth (265).

In conclusion, re-educating TAMs to limit their pro-tumour activities and/or potentiate their anti-tumour functions may represent an effective strategy, either as monotherapy or in combination with other antineoplastic therapies or immunotherapies.

1.7 Experimental tools to investigate ontogeny, maintenance and function of macrophages in healthy and malignant tissues

1.7.1 Lineage tracing mice

Recent advances in lineage tracking techniques have provided new insights into the origins and fate of macrophages in mice, leading to revise the ontogeny dogma for the "mononuclear phagocyte system". With the currently available fate mapping tools it is possible to permanently label in vivo specific cell subsets, and the consequent progeny, expressing a genetically recombinant reporter gene, for instance Yellow fluorescence protein (YFP), that is under the control of a constitutive ubiquitous promoter – usually Rosa26 (266). The reporter transgene is transcribed upon tamoxifen-inducible activation of a genetically encoded site-specific recombinase, which is expressed by
the cell population of choice. Hence, the cell type-specific recombination enables the labelling of embryonic precursor cells at precise time windows of the embryogenesis and track them into adulthood. In Cre recombinase-dependent fate mapping models, Cre transgene is encoded under the control of an endogenous promoter that is expressed by the cell population of interest. The floxed reporter transgene is then constitutively activated upon induction of the recombinase, which removes the stop-flox cassette.

An advantage of this approach is the irreversible tagging of the precursor of interest and its progeny, however the Cre recombinase expression depends on a specific promoter, which may not be exclusively expressed on a cell type or may be expressed at insufficient levels, so that Cre recombinase levels are not enough to efficiently cut the stop-flox cassette. Moreover, for time constraints Cre recombination might not occur in rapidly dividing progenitors. Thus two possible weaknesses of this system might be its partial selectivity and incomplete labelling (267).

Several fate mapping models have enabled the tracking of different embryonic macrophage populations into adulthood, taking advantage of the differential expression of transcription factors and surface markers (Figure 1.17 and Table 4). In the inducible Runt-related transcription factor 1 (Runx1CreER) system, the expression of Runx1 in the YS at about E7.0, before the onset of definitive hematopoiesis (268), was chosen to track E7.0 Runx1-expressing YS-derived macrophages, to demonstrate that they persist into adulthood as microglia. Indeed, labelled primitive progenitors migrated from the YS to the brain between E8.5 and E9.5 and became the seed population for microglia (42). Using the same system it has been feasible to distinguish two populations of skin Langerhans cells, YS-derived and fetal monocyte-derived, with the latter prevailing over time (42,46).

The inducible Cs1CreER system has been utilized to induce the labelling at E8.5, confirming that early EMPs-derived YS macrophages give rise to microglia (32,44). Injection of tamoxifen at E8.5 or E9.5 does not efficiently label late EMPs or FL monocytes, as opposed to early EMPs. The E8.5 pulse-labelling of Csf1r-expressing precursors has thus provided evidence that CSF1R can be a robust marker of YS-derived tissue-resident macrophages. YFP expression was not detected in the progenies of adult HSCs, however macrophages in adult organs such as heart and liver retained YFP+ YS-derived macrophages, even though late EMP-derived FL monocytes are considered to be the main precursor of adult macrophage populations as they outcompete early EMP-derived YS macrophages (32,37,55). This suggests that Cs1CreER has a longer window compared to Runx1CreER and it cannot be excluded that YS progenitors migrating to the fetal liver at later stages of embryogenesis may
continue to give rise to adult tissue macrophages. The contribution of EMPs (whether early or late) to adult tissue-resident macrophages remains debated; for instance it has also been proposed that FL HSCs may be the precursors (40). In a tamoxifen-inducible \( \text{Kit}^{\text{MerCreMer}} \) model it is possible to tag cells that express the stem-cell factor receptor c-Kit, and their progeny. E8.5 tamoxifen injection labeled all adult peripheral hematopoietic cells including adult tissue-resident macrophages. These results led to the hypothesis that adult macrophages, with the exception of microglia and partially Langerhans cells, arise from definitive HSC-derived FL monocytes (40). Nevertheless, it has been pointed out that both EMPs and fetal HSCs express c-Kit and co-exist in the FL until E16.5 (37, 269), hence \( \text{Kit}^{\text{MerCreMer}} \) system may not be optimal to distinguish between late EMPs and fetal HSCs (38).

<table>
<thead>
<tr>
<th>Fate mapping marker</th>
<th>Marker-expressing cells</th>
<th>Labelling outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX1</td>
<td>Early EMPs</td>
<td>Identification of YS-derived embryonic macrophages in adulthood (microglia, Langerhans cells)</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Early EMPs</td>
<td>Corroboration of YS-derived origin for microglia (persistence of labelling from YS-derived macrophages in the embryo to adult microglia). Majority of tissue-resident macrophages derive from definitive late EMPs</td>
</tr>
<tr>
<td>KIT</td>
<td>HSCs</td>
<td>Corroboration of YS-derived origin for microglia. Majority of tissue-resident macrophages derive from definitive fetal HSCs</td>
</tr>
<tr>
<td>FLT3</td>
<td>HSCs</td>
<td>Identification of adult macrophages generated by definitive haematopoiesis (HSCs)</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3CR1(^+) cells</td>
<td>Labelling of cells that pass through a CX3CR1(^+) stage</td>
</tr>
</tbody>
</table>

**Figure 1.17 and Table 4:** Fate mapping tools utilized to define macrophage lineages. Schematic illustration summarizing some of the lineage tracing tools that enabled to define adult macrophage ontogeny, when induced at precise time windows during embryogenesis (\( \text{Runx1}^{\text{CreER}}, \text{Csf1r}^{\text{CreER}}, \text{Flt3}^{\text{Cre}} \)). No tools are currently available to distinguish fetal liver-derived monocytes, hence to trace FL monocytes. Figure taken from Epelman S., Lavine K.L., Randolph G.J. Immunity. 41(1): 21–35 (2014).
Introduction

The Flt3\textsuperscript{Cre} system takes advantage on the transient expression of Flt3 in adult definitive HSCs during the differentiation into all lineages, i.e. myeloid, lymphoid, megakaryocyte, and erythroid cells (270). It has been shown that Flt3-Cre\textsuperscript{+} blood monocytes do not replace Flt3-Cre\textsuperscript{−} tissue-resident macrophage populations in the adulthood (44,48,55). These latter may be purely YS-derived or may consist of a mixed population containing macrophages derived both from both the YS and from fetal monocytes (55).

The Cx3cr1\textsuperscript{Cre} fate mapping system labels all those cells that pass through a CX3CR1\textsuperscript{+} differentiation stage, including myeloid cells of the central nervous system (CNS), circulating monocytes, peripheral macrophages and dendritic cells as well as myeloid progenitors in the BM. All of these cells become YFP\textsuperscript{+} after the initial tamoxifen pulse, whereas HSCs are not labelled because do not express CX3CR1, thus not affected by the tamoxifen-induced recombination. After a four-week chase period, no more YFP\textsuperscript{+} cells can be detected in the circulation, whereas CNS resident macrophages retain the labelling (271).

1.7.2 Impairment of recruitment/survival axes

The local cytokine milieu is important for the regulation, maintenance and functional specialization of macrophages. To examine whether CSF1 is required for the macrophage homeostasis in peripheral tissues, mice models as well as in vivo techniques have been developed. Mice with a spontaneous null mutation in Csf1 (Csf1\textsuperscript{op/op} mice) - known as osteopetrotic mice - have a broad reduction of tissue-resident macrophage populations (113,272), on the contrary microglia numbers are only slightly reduced (42). It has been described that mice with targeted ablation of Csf1r (Csf1r-null mice) have a more severe phenotype than that of the Csf1\textsuperscript{op/op} mice, including the complete loss of microglia and Langerhans cells (42, 273). This suggests that other ligands other than CSF1 binds CSF1R. Indeed, IL-34 is an additional ligand for CSF1R and is mainly expressed in the brain and epidermis (274). IL-34-deficient mice have shown a drastic reduction of microglia in the brain regions in which IL-34 is normally produced (275), suggesting that IL-34 may compensate for the loss of CSF1 in Csf1\textsuperscript{op/op} mice. Despite of the importance of CSF1R in macrophage differentiation, Csf1r-null mice still have some tissue macrophages, such as in the spleen, indicating the existence of other macrophage growth factors. In order to investigate the role of tissue-resident macrophages under steady state and pathological conditions, CSF1R signaling blockade can be achieved through anti-CSF1R antibody injection. For instance, the monoclonal antibody RG7155 binds CSF1R and potently inhibits the viability of macrophages (246).
Other than CSF1, cytokines such as CSF2 have been proposed to sustain macrophage maintenance. However, in mice lacking CSF2 only alveolar macrophages are drastically impaired, which indicates that CSF2 has a key role in the homeostasis of lung macrophages (48).

In organs such as intestine, in mice lacking either CCR2 or its ligand CCL2 tissue-resident macrophages were markedly reduced, indicating that macrophage maintenance requires the CCR2-dependent recruitment of monocytes to the adult mucosa (58). CCR2-deficient mice are a useful tool to investigate the dependency on blood monocytes, as the numbers of Ly6C\[^{hi}\] blood monocytes are diminished because they require CCR2 to exit the BM (276). In wild-type (WT) mice the impairment of CCR2-CCL2 axis can also be obtained through the injection of CCL2 neutralizing antibody (nAb) (277).

### 1.7.3 Mixed bone-marrow chimeric mice

Mixed bone marrow chimeric mice can be used to investigate BM-dependency of tissue-resident macrophages in steady state and perturbed tissues. A chimeric mouse is generated by BM irradiation and engraftment of the BM that belongs to a donor mouse with different genotype. Upon transfer of donor BM-cells into the irradiated recipient, over 90% of circulating monocytes are usually of donor origin. An aspect to consider is the radiation-induced damage of certain anatomical barriers, including the BBB (278), which results in the spontaneous entry of BM-derived monocytes. This approach may introduce the artificial engraftment of donor monocytes that can differentiate into macrophages, even in “closed” tissues such as brain (44).

### 1.7.4 Parabiosis

BM-contribution to specific tissue-resident macrophage populations can been investigated in parabiosis experiments. Studies of parabiosis have further corroborated that in majority of the adult tissues BM-derived progenitors do not refill macrophage populations (48). Parabiotic mice are surgically joint by cutaneous vascular anastomoses so that they share a common blood circulation while maintaining separate organs and tissues. If monocytes give rise to tissue-resident macrophages in the steady state, the macrophage chimerism in the parabiont should be comparable to the monocyte chimerism in blood. Apart from the technical challenge, a major drawback of this model is the low level of chimerism – 15-30% chimerism for blood Ly6C\[^{hi}\] monocytes (279) – that is directly related to the half-life of blood-borne cells in the circulation. This may cause an underestimation of infiltrating cells (280).
1.7.5 In vivo conditional cell ablation with diphtheria toxin receptor-diphtheria toxin (DTR-DT) system

The diphtheria toxin receptor-diphtheria toxin system (DTR-DT) is a useful tool to selectively ablate a specific cell subset in vivo. In DTR transgenic mice the transgene encoding for human DTR (hDTR) is controlled by a promoter of choice, and in this case a promoter for a cell-specific protein. Hence, DTRs are expressed only in the cell type of interest (281), and upon DT injection exclusively that cell type undergoes cell death. In detail, DT consists of two subunits; binds the hDTR through the B subunit (DT-B), and is incorporated into the cell by receptor-mediated endocytosis. The A subunit (DT-A) inhibits protein synthesis by inactivating elongation factor 2 (EF-2), and subsequently induces cell death. The DT-A is highly toxic, since one molecule in the cytosol may be enough to kill the cell (282).

A few DTR-DT transgenic mice have been generated to investigate macrophage functions. In lysozyme M promoter-directed Cre/iDTR (lysM-Cre/iDTR) mice (283,284), Cre recombinase leads to DTR expression in lysM+ cells, including macrophages. LysM+ macrophages are thus efficiently (83-98%) ablated upon DT administration; however this system is in not specific since it fully depletes the granulocytes. Goren et al. used lysM-Cre/iDTR mice to investigate macrophage functions in skin repair, as DT injection led to a rapid reduction in both skin and wound macrophage numbers at sites of injury (285). The same transgenic mouse was utilized by Lee et al. to study the role of macrophages in energy homeostasis (286).

In Cx3cr1CreER;IDTR mice, tamoxifen-induced Cre-mediated recombination leads to the expression of DTR in CX3CR1-expressing cells. This system was established to specifically deplete microglia from the brain after DT administration, leaving CX3CR1+ cells in the periphery unaffected (65,287).

Recently a CD169-DTR transgenic mouse has been used to unravel the function of tissue-resident macrophages in experimental malaria (288). CD169 is a lectin-type receptor specifically expressed in F4/80hi tissue-resident macrophages, whereas not detected in infiltrating monocytes and F4/80int monocyte-derived macrophages as well as neutrophils. In this DTR-DT system, CD169+ tissue-resident macrophages express the transgene hDTR and are specifically ablated upon DT injection, hence it is possible to study the role of these cells in vivo, in different organs, both under steady state and pathological settings (288,289).

1.7.6 Mouse models in oncoimmunology

Mouse models of transplantable, carcinogen-induced or genetically engineered cancers have been – and currently are – useful in vivo tools to understand fundamental
mechanisms of tumorigenesis as well as the multifaceted interplay between cancerous cells and the tumour microenvironment. Thanks to the mouse models it has been possible to describe the so-called process of cancer immunoediting, wherein tumour-educated leukocytes can exert tumour-promoting functions.

Hereafter, three main tumour models will be described:

1. Transplantable tumours;
2. Carcinogen-induced tumours;
3. Spontaneous tumours in genetically engineered mice - and the Apc\textsuperscript{Min/+} mouse model as example of spontaneous cancer in the gut.

1) Transplantable tumours
A widely used mouse model consists of the inoculation of histocompatible cancer cell lines into immunocompetent mice (290). The cancer cell line is commonly injected subcutaneously into the flank of the mouse, or eventually either orthotopically and systemically. Compared to subcutaneous tumours, orthotopic tumours grow in a more “realistic” environment. The systemic injection of tumour cells (both i.p. and i.v.) may allow studies of metastatic spread.

Even though mouse models of transplantable tumours are a valid alternative to genetically engineered mouse models for their relatively limited cost, they are still considered “imperfect” in recapitulating the disease. Firstly, transplantable tumours derive from a cell line that is obtained in vitro and lacks of the genetic heterogeneity that characterizes spontaneous tumours. On top of that, several injected cells may undergo cell death shortly after injection, producing an initial vaccination effect. Moreover, after inoculation the tumour rapidly grows eluding the classical multi-step carcinogenesis, and it is not characterized by the chronic inflammatory microenvironment that is created in natural settings (291-293).

2) Carcinogen-induced tumours
Several carcinogens can be utilized to generate a “natural” tumorigenesis in mice. For instance, local application of ultraviolet (UV) light induces squamous cell carcinomas, whereas methylcholanthrene (MCA) induces fibrosarcomas. The combination of “initiator” 7,12-dimethylbenz[a]anthracene (DMBA) and “promoter” 12-O-tetradecanoylphorbol-13-acetate (TPA) causes papillomas that eventually evolve to squamous cell carcinomas (294). Breast cancer can be obtained by combining DMBA and the stimulation of progesterone receptor (295). Colorectal cancer (CRC) is usually induced through a combination of azoxymethane (AOM) and dextran sodium sulfate (DSS) that cause DNA damage and inflammation, respectively (296). Lung cancer can
be obtained with urethane and hepatocellular carcinoma with N-nitrosodiethylamine (DEN).

Compared to transplantable tumours, chemically induced tumours have the advantage that they gradually grow, developing an intrinsic heterogeneity. They are more “realistic” because genetically diverse and bearing the chronic inflammation. In these settings, it is possible to study the contribution of the immune system in the tumorigenesis. Nevertheless, a chemically-induced tumour model is more problematic to handle since the variability in the tumour onset and development between mice may create issues in the data interpretation, especially in small cohort experiments (293).

3) Spontaneous tumours in genetically engineered mice

Genetically engineered mice develop tumours that are generated by overexpression of oncogenes or inactivation of tumour suppressor genes through genetic recombination, and accurately reflect human tumours. Since the genetically induced tumour progression mimics a realistic scenario, it has been possible to achieve important insights regarding the crosstalk between cancer and the immune system. Nevertheless, as for the chemically induced tumour models, it may take a long period of time for tumours to progress, and the tumour monitoring can be an issue (293). With regards to genetically engineered mouse models of colorectal tumorigenesis, the Apc\textsuperscript{Min/+} mouse is widely used since it spontaneously develop intestinal tumours that mimic the multistep process of tumorigenesis. The Apc\textsuperscript{Min/+} mouse carries a heterozygous mutation at codon 850 of “Adenomatous polyposis coli” (Apc) gene. The mutated allele, referred as “Multiple intestinal neoplasia” (Min) (297), results in a truncated protein lacking the C-terminal domain. In the Wnt intracellular pathway, APC protein forms a complex with Axin and helps Glycogen synthase kinase 3 beta (GSK3β) to phosphorylate the N-terminal serine/threonine residues of β-catenin, accelerating its rapid degradation through ubiquitination (298). If APC is truncated, GSK3β cannot phosphorylate β-catenin, which accumulates in the cytoplasm and translocates into the nucleus where it activates T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), inducing the transcription of Wnt target genes. This leads to cell proliferation and differentiation of the intestinal epithelium (299,300). Apc is classified as a recessive tumour suppressor gene, and inactivation of both alleles is essential for tumour development. Although the condition of homozygosity Apc\textsuperscript{Min/Min} is embryonically lethal, Apc\textsuperscript{Min/+} mice are spontaneously predisposed to develop numerous adenomas and infrequent carcinomas in the small intestine by 4-5 months of age along with rare colon adenomas and carcinomas (301) as a result of the loss of heterozygosity (LOH) in Apc (302). Of note, Apc\textsuperscript{Min/+} mice generally develop benign adenomas, predominantly in the
small intestine, whereas most human intestinal cancers tend to arise in the colon and demonstrate aggressive invasion or metastasis (303). The majority of Apc<sup>Min/+</sup> mice die at about 6 months old due to anemia, cachexia, and eventually intestinal obstruction. At that age they all have splenomegaly and rare cases of female Apc<sup>Min/+</sup> mice may develop mammary tumours. Apc<sup>Min/+</sup> mice can be used in combination with colitis-associated treatments in order to accelerate the process of tumour development. It is widely well-recognized that inflammation enhances the risk of CRC (304) and inflammatory stimuli induced by DSS have been shown to strongly promote colon tumorigenesis in Apc<sup>Min/+</sup> mice. DSS is a negatively charged polymer of glucose with inserted sulfate groups; it associates with medium chain length fatty acids, enriched principally in the distal colon lumen. These complexes form vesicles that are able to fuse with the membrane of colonic epithelial cells. Once in the cytoplasm, these vesicles affect major epithelial cell pathways, leading to the disruption of the intestinal epithelial barrier (305). This damage in turn enables luminal antigens and bacteria to invade the LP, and the subsequent enhanced inflammatory response. In Apc<sup>Min/+</sup> mice, 1-week DSS treatment induces multiple colonic neoplasms by 4 weeks post-treatment. In WT mice, the same treatment does not induce neoplastic lesions, indicating that one cycle of DSS is not sufficient to trigger the tumour formation in absence of genetic predisposition. Even though the inflammatory mechanisms supporting the intestinal tumorigenesis are not fully explained yet, during and after DSS treatment several immune cells are recruited in the damaged tissue and secrete soluble factors, including ROS, RNS, COX2 and iNOS, which in turn promote the development of colonic neoplasms. After the tumour onset, the chronic production of inflammatory mediators will likely fuel the progression of colitis-associated CRC (306).

1.8 Objectives of the project

The tumour microenvironment - an intricate network of neoplastic cells, infiltrating immune cells and ECM - orchestrates cancer initiation and progression. Among the myeloid cell compartment, the origins and maintenance of TAMs in colon cancer have not investigated thus far. Furthermore, in vivo functional studies in colon cancer models are still lacking and clinical studies of correlation between TAM abundance and prognosis have given conflicting results. In light of the above, the objectives of this project can be summarized as follows:

1. To characterize the myeloid and lymphoid cell compartment in murine colon adenomas;
2. To confirm the lineage of intestinal macrophages and in parallel to define the ontogeny and maintenance requirements of tumour-resident macrophages.

3. To investigate whether TAMs are involved in the tumour progression in a preclinical mouse model of colon cancer.

These aims were pursued taking advantage of various in vivo approaches:

- genetically-induced colon cancer model (\( APC^{Min/+} \) mouse);
- fate mapping mouse model (\( Kit^{Mercremer/R26} \) mouse);
- constitutive and conditional impairment of cytokine axes (CCR2-deficient mouse and CSF1R blockade);
- conditional ablation of resident macrophages (CD169-DTR mouse and CSF1R blockade).
2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Apc\textsuperscript{Min+/+} mouse strain (C57BL/6J-Apc\textsuperscript{Min/J}) (The Jackson Laboratory, Bar Harbor, ME USA) was kindly provided by Dr. Lai Guan Ng (Singapore Immunology Network, A*Star). Kit\textsuperscript{MerCreMer/R26} mice were generated in our laboratory as previously described (40). Ccr\textsuperscript{2−/−} mice (B6.129S4-Ccr\textsuperscript{2\textsuperscript{mTm1ld}}/J) (307), Csf2r\textsuperscript{−/−} mice (B6.129S1-Csf2rb\textsuperscript{tm1Cgb/J}) (308) C57BL/6J and C57BL/6J Cd45.1\textsuperscript{+} (B6.SJL-Ptnrc\textsuperscript{a/Pepc\textsuperscript{b}/BoyJ) mice were purchased from The Jackson Laboratory (USA). C57BL/6J, C57BL/6J Cd45.1\textsuperscript{+} and C57BL/6J Cd45.1\textsuperscript{+/Cd45.2\textsuperscript{+}} were bred in our facility. CD169-DTR mice were generated in our laboratory, as previously described (289). Cx3cr1-gfp mice were generated by Dr. Dan R. Littman (New York University Medical Center) and kindly provided by Dr. Florent Ginhoux (Singapore Immunology Network, A*Star). Apc\textsuperscript{Min+/+} mice were crossed with Kit\textsuperscript{MerCreMer/R26} and Ccr2\textsuperscript{−/−} mice to obtain Kit\textsuperscript{MerCreMer/R26}Apc\textsuperscript{Min+/+} and Ccr2\textsuperscript{−/−}Apc\textsuperscript{Min+/+} mice respectively. Apc\textsuperscript{Min+/+} mice were crossed with CD169-DTR and Csf2r\textsuperscript{−/−} mice to obtain CD169-DTR Apc\textsuperscript{Min+/+} and Csf2r\textsuperscript{−/−}Apc\textsuperscript{Min+/+} mice respectively. Apc\textsuperscript{Min+/+} mice were crossed with CD169-DTR Kit\textsuperscript{MerCreMer/R26} mice to obtain CD169-DTR Kit\textsuperscript{MerCreMer/R26}Apc\textsuperscript{Min+/+} mice. Germ free C57BL/6J mice were kindly provided by Prof. Sven Pettersson (Lee Kong Chian School of Medicine, Nanyang Technological University). All mice were bred and housed under specific-pathogen-free (SPF) conditions in the animal facility of Nanyang Technological University. For the experiments, both genders were used and equally distributed within experimental and control groups. This study was carried out in strict accordance with the recommendations of the NACLAIR (National Advisory Committee for Laboratory Animal Research) guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore. All experiments were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University.

2.1.2 Media, buffers and solutions

Media, buffers and solutions are listed in Appendix 6.1.

2.1.3 Reagents, chemicals and kits

Reagents, chemicals and kits are listed in Appendix 6.2.

2.1.4 Equipment

Equipment used in this study is listed in Appendix 6.3.
2.1.5 Software
Flow cytometry data were analysed using FlowJo 7.6.1 software (TreeStar Inc, Ashland, OR). Graphs and statistical analysis were generated with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

2.2 Methods

2.2.1 Genotyping
5-mm tail was digested with proteinase K (1:100) in SNET buffer (final volume of 500 μl) at 55°C, 950 rpm, overnight. After centrifugation the supernatant was mixed with 650 μl isopropanol to precipitate the DNA, which was subsequently centrifuged and washed with ethanol 75%. The DNA pellet was resuspended in 1X TE buffer and incubated at 65°C, 950 rpm, 1 hour. 1-2 μl of DNA was added to the PCR mastermix containing 0.5 μl dNTPs, 5X GoTaq buffer, 2 μl MgCl₂, 1 μl primer forward and 1 μl primer reverse (primers listed in Appendix, Table 10), 0.3 μl DNA Taq polymerase, dH₂O to volume (25 μl).

2.2.2 Induction of intestinal tumours
8-week-old Apc<sup>Min/+</sup> mice and the wild-type littermates, were given 1.5% (w/v) DSS in autoclaved drinking water, ad libidum for 7 consecutive days, with fresh DSS supplied at day 4. At day 8, the DSS was replaced with water. The body weight was monitored daily from day 7 until body weight recovery, and every 2-3 days till the end of the experiment (day 35-40). The DSS dosage was adjusted in order to induce 5-10% of body weight reduction post-DSS treatment and a complete body weight recovery by day 14, to ensure a moderate DSS-induced colitis enough to trigger the polyp formation. Mice were terminated when reaching the analysis timepoint or when showing symptoms of weight loss and/or other signs of physical discomfort (e.g. lethargy, rectal prolapse, severe bleeding).

2.2.3 Tamoxifen-inducible fate mapping
In the adult labelling, Kit<sup>MerCreMer/R26</sup> and Kit<sup>MerCreMer/R26</sup>Apc<sup>Min/+</sup> mice were administered 5 doses of tamoxifen, 4 mg/dose, for 5 consecutive days by gavage with a feeding needle. In the embryonic labelling, pregnant female mice at E10.5 were administered tamoxifen through a single i.p. injection of 4 mg tamoxifen in 300 μl corn oil. In the adult fate mapping experiment, after tamoxifen administration to Kit<sup>MerCreMer/R26</sup> mice and Kit<sup>MerCreMer/R26</sup>Apc<sup>Min/+</sup> mice, c-kit<sup>+</sup> BM-derived HSCs become labelled with YFP and all cells deriving from YFP<sup>+</sup> BM-HSCs permanently maintain YFP expression. This effect
enables to distinguish whether adult intratumoural macrophages are derived from adult BM definitive haematopoiesis or from embryonic haematopoiesis. Tamoxifen was administered one month after inducing the tumour formation through DSS. In order to obtain a full YFP labelling of BM-derived cells, which depends on the cell turnover within the tissue, tumours and healthy LP were harvested 7 weeks after the last tamoxifen dose (for more details, refer to Figure 3.21 and Figure 3.24a in the Results section). In the embryonic fate mapping experiment, tamoxifen injection was performed at E10.5 in order to label c-kit+ FL-derived precursors, and consequently their progenies. After birth, 8-week-old Kit\textsuperscript{MerCreMer/R26} Apc\textsuperscript{Min/+} mice were subjected to DSS treatment to induce the tumours. The YFP analysis was performed one month later (for more details, refer to Figure 3.21 and Figure 3.24b in the Results section). Except those macrophages derived from embryonic primitive haematopoiesis, colon LP and intratumoural macrophages are expected to be YFP labelled.

### 2.2.4 DT-mediated ablation

For systemic ablation of CD169\textsuperscript{+} tissue-resident macrophages, CD169-DTR Apc\textsuperscript{Min/+} and CD169-DTR Kit\textsuperscript{MerCreMer/R26} Apc\textsuperscript{Min/+} mice were i.p. injected twice for 2 consecutive days with 20 ng/gbw DT in 1X PBS, containing 1% mouse serum. Depletion efficiency of intratumoural CD169\textsuperscript{+} tissue-resident macrophages was analysed in CD169-DTR Apc\textsuperscript{Min/+} mice the day after the second injection. The effective ablation of CD169\textsuperscript{+} macrophages, both in tumours and in healthy LP, enables to determine whether BM-derived macrophages can fully refill their cell pool within each tissue. CD169-DTR Apc\textsuperscript{Min/+} and CD169-DTR Kit\textsuperscript{MerCreMer/R26} Apc\textsuperscript{Min/+} mice were first subjected to DSS treatment; one month later tamoxifen was administered for 5 consecutive days to induce the tracing of BM precursors. After one month, DT-mediated macrophage ablation was performed. Four weeks after the DT treatment, tissues were harvested to analyse the percentage of YFP\textsuperscript{+} cells (for more details on the experimental protocol, refer to Figure 3.28 in the Results section).

### 2.2.5 Mixed bone marrow chimeric mice

At day 1, recipient congenic C57BL/6 Cd45.1\textsuperscript{+}/Cd45.2\textsuperscript{+} mice were lethally irradiated through a total of 11 Gy radiation administered 4 hours apart in two separate doses of 5.5 Gy, using a BioBeam gamma irradiation device. At day 2, BM cells from C57BL/6 Cd45.1\textsuperscript{+} mice were harvested and incubated 10 minutes at room temperature with 0.89% ammonium chloride to induce the red blood cell lysis. After filtration through a 40 μm nylon mesh, 3x10\textsuperscript{6} CD45.1\textsuperscript{+} BM cells were intravenously injected through the
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retro-orbital vein in recipient mice. Ten weeks after reconstitution, the colon LP was processed and analysed.

2.2.6 Polyp count
The dissected colon was opened longitudinally and washed in 1X PBS to remove the luminal contents. Colonic polyps were macroscopically counted and with the help of a sliding caliper they were categorized as > 2 mm (large) and < 2 mm (small).

2.2.7 Colon histology
At day 35, dissected colons from DSS-treated Apc\textsuperscript{Min/+} mice were opened longitudinally and washed in 1X PBS. Swiss-roll folded colons were fixed in organ fixation buffer for 24-48 hours, subsequently dehydrated in 3 incubations steps with increasing concentration of ethanol: 80% ethanol for 2 hours, 90% ethanol for 2 hours, 100% ethanol for 4 hours. Dehydrated colons were subjected to the following clearing steps: ethanol:xylene (1:1) for 30 minutes, xylene for 3 hours. Colons were transferred in liquefied paraffin, incubated over night at 65°C, and embedded in paraffin cassettes for sectioning. 5 μm sections were placed on microscope slides, deparaffinized twice in xylene for 15 minutes, subsequently rehydrated through the following steps: 95% ethanol, 70% ethanol and distilled water for 5 minutes each. Tissue sections were stained in hematoxylin solution for 30 minutes, washed in running tap water, differentiated in 1% acid ethanol solution for 30 seconds, washed for 1 minute and transferred in Scott's tap water substitute for 1 minute (bluing step). Tissue sections were washed for 5 minutes, rinsed in 95% ethanol for 1 minute, counterstained in eosin solution for 15 seconds, dehydrated in 90% and 100% ethanol for 10 minutes respectively. Slides were cleared in xylene for 10 minutes and mounted using a xylene-based mounting medium (DPX Mountant for histology) to be visualized by light microscopy with 4X, 10X and 20X magnification for image capture.

2.2.8 Colon immunohistochemistry
Dissected colons were opened longitudinally, washed in 1X PBS, swiss-rolled and placed onto dedicated molds containing the Optimal Cutting Temperature (OCT) compound, and immediately transferred on dry ice, to be stored at -80°C until sectioning. 8 μm sections were placed on glass microscope slides and air-dried for 1 hour. The slides were fixed in cold acetone for 10 minutes and rehydrated in 1X PBS for 5 minutes. Blocking of non-specific binding was obtained by incubation with Fc blocking solution for 30 minutes at 4°C, followed by washing with 1X PBS. Sections were stained overnight at 4°C with antibodies diluted in blocking buffer with 0.1%
sodium azide (antibodies are listed in Appendix, Table 7). Stained sections were then washed in 1X PBS and incubated with DAPI (1:1,000 in PBS 2%) for 10 minutes. After washing in 1X PBS, slides were mounted with fluorescence mounting medium and visualized by fluorescent microscopy using a 20X magnification for image capture.

2.2.9 Isolation of mononuclear cells from colon lamina propria
Colon LP cell suspension was obtained as previously described in (309). Dissected colons were opened longitudinally, washed in 1X PBS to remove the luminal contents. To separate the epithelial layer, colons were transferred in 25 ml 1x PBS with 1.3 mM EDTA and shaked at 37°C, 370 rpm for 60 minutes. Colons were subsequently washed in digestion medium, minced with the help of dissecting scissors and digested with 0.1 mg/ml Collagenase D and 2 U/ml DNase I in 10 ml digestion medium at 37°C, 370 rpm, for 90 minutes. Single cell suspension was obtained by mashing the digested tissue through a 100 μm cell strainer and debris removal through 70%-40% Percoll™ gradient, at 2800 rpm for 10 minutes. The mononuclear cells, localized at the gradient interface, were transferred into a new tube with 10 ml of PBS 2%. The cell suspension was then centrifuged at 500 g for 5 minutes to obtain the cell pellet for flow cytometry.

2.2.10 Isolation of mononuclear cells from tumour
Colon polyps were manually isolated with the use of curved forceps, minced and digested with 0.1 mg/ml Collagenase D and 2 U/ml DNase I in 2 ml digestion medium at 37°C, 900 rpm, for 60 minutes. Digested tissues were mashed through a 100 μm cell strainer to obtain a cell suspension. The mononuclear cell population was enriched through centrifugation in 35% Percoll™ at 700 g for 10 minutes. The obtained cell pellet was then labelled for flow cytometry.

2.2.11 Cell labelling for flow cytometry
Cells isolated from the tissues were incubated with Fc block at 4°C for 15 minutes. For staining of cell surface antigens, after one wash with PBS 2% blocked cells were stained with a combination of fluorochrome-conjugated monoclonal antibodies diluted in PBS 2%, at 4°C for 20 minutes (antibodies are listed in Appendix, Table 7). For intracellular staining of ARG1, cells were washed once after extracellular staining, hence fixed and permeabilized using Cytofix/Cytoperm™ Kit according to the manufacturer’s instructions before incubation with the intracellular antibody in 1X Foxp3 Permeabilization buffer, RT for 30 minutes. For intracellular staining of Foxp3 and Ki67, the cells were processed following the manufacturer’s instructions of Foxp3 Fixation/Permeabilization Kit. Stained cells were washed once and subsequently
resuspended in PBS 2% for flow cytometry analysis using 5-laser Fortessa™ X-20 cell analyser.

2.2.12 Cell sorting
Cells were stained and then filtered through a 40 μm nylon mesh. Cell sorting was performed with a 100 μm nozzle in FACSARia™ cell sorter. Sorted cells were eluted in RNase free 1X PBS, subsequently Giemsa stained and visualized by light microscopy or directly lysed for total RNA extraction.

2.2.13 Morphology visualization of sorted cells
Sorted cells were transferred onto microscope glass slides using the Cytospin™ equipment according to the manufacturer’s protocol. Cells were subjected to the following steps: fixation with methanol for 1 minute, 1X Giemsa staining for 20 minutes, rinsing in deionized water for 3 times. The slides were then mounted with DPX mounting medium and visualized by light microscopy using a 100X magnification.

2.2.14 RNA sequencing and transcriptome analysis
Total RNA was extracted using the Arcturus™ PicoPure™ RNA Isolation Kit according to the manufacturer’s protocol. All RNA samples were tested with an Agilent Bioanalyser for quality assessment. Complementary DNA (cDNA) libraries were prepared from 2 ng total RNA starting material and 1 μl of a 1:50,000 dilution of ERCC RNA Spike in Controls using the SMARTSeq v2 protocol (310) with the following modifications: 1) addition of 20 μM TSO; 2) use of 250 pg cDNA with 1/5 reaction of Illumina Nextera XT kit. The length distribution of the cDNA libraries was monitored using a DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip. All cDNA samples were subjected to an indexed paired-end sequencing run of 2 X 51 cycles on an Illumina HiSeq 2500 system (Illumina) under rapid run mode (17 samples/lane). The paired-end reads were mapped to the Mouse GRCm38/mm10 reference genome using the STAR alignment tool (311). Mapped reads were summarized to the gene level using featureCounts (V1.5.0-P1) software (312) and with GENCODE gene annotation (313). Genes with an average number of reads per sample <10 in all cell subpopulations were filtered out from subsequent analyses. For differentially expressed gene (DEG) analysis, the limma/voom pipeline was used as recommended by the MicroArray Quality Control (MAQC) project (314) as one of the best performing RNA-seq data analysis pipelines. Different cell populations were compared using limma and DEGs were selected with Benjamini–Hochberg adjusted P-values < 0.05.
Hierarchical clustering and principal component analysis were performed with Log2 transformed value of RPKM (Reads Per Kilobase of transcript per Million mapped reads). All analyses were performed in R version 3.1.2 (URL http://www.R-project.org/). The web server Heatmapper (URL http://www.heatmapper.ca/) (315) was also used to obtain the heat maps. The Volcano plots of DEGs between different cell groups were generated with GraphPad Prism 6 software. RNA sequencing and bioinformatical analysis were kindly performed by Shihui Foo and Kaibo Duan, respectively (Singapore Immunology Network, A*Star).

2.2.15 RNA isolation from tissue bulk and reverse transcription to cDNA
For mRNA target quantification from tumour bulk and LP bulk, single polyps and distal colons (distal portion, last 2 cm) were minced in 1 ml of Trizol™ and homogenized. Total RNA was extracted using RNAsimple Total RNA kit following the manufacturer’s protocol. Complementary DNA (cDNA) was generated with 2 μg of RNA using oligo(dT) primers, dNTP Mix, RNAse Inhibitor, RNAse free water, M-MLV RT Reaction Buffer 5X and M-MLV Reverse Transcriptase according to the manufacturer’s instructions.

2.2.16 RNA isolation from FACS-sorted cells and reverse transcription to cDNA
For mRNA target quantification from FACS-sorted cells, total RNA was extracted using Arcturus™PicoPure™ RNA Isolation Kit, quantified using Quant-iTTM RiboGreen® RNA Reagent and Kit, subsequently retro-transcribed and amplified with Ovation® Pico WTA System V2 Kit. RNA isolation, quantification and production of amplified cDNA were performed according to the manufacturer’s protocol.

2.2.17 Quantitative real-time PCR
Quantitative real-time PCR was performed with 20 ng of cDNA per reaction with 0.5 μM forward and reverse primers and FAST 2x qPCR Master mix (primers are listed in Appendix, Table 11). For the analysis, all reactions were run in triplicate. The threshold cycle (Ct) value for gene being tested was normalized to the Ct value for housekeeping gene (β-actin) to obtain ΔCt. The value of $2^{-\Delta\Delta Ct}$ was calculated to obtain the expression fold change between groups (LP versus tumour of different sizes; LP MHCIIhi macrophages versus LP MHCIIlo macrophages, intratumoural MHCIIhi macrophages and MHCIIlo macrophages; intratumoural monocytes versus intratumoural MHCIIhi macrophages and MHCIIlo macrophages).
2.2.18 Statistical analysis

Data were analysed with GraphPad Prism 6 software. All values are expressed as the mean ± s.e.m. Samples were analysed by unpaired Student’s t-test (two-tailed) or Bonferroni two-way analysis of variance (ANOVA). A P-value of <0.05 was considered to be statistically significant.
3 RESULTS

3.1 Characterization of colonic adenomas

3.1.1 Apc\(^{\text{Min/+}}\) mice spontaneously develop intestinal tumours

Apc\(^{\text{Min/+}}\) mice carry a heterozygous mutation in the gene Apc, hence are genetically inclined to develop intestinal cancer. As previously reported \((297,301,302)\), we were able to confirm that 4-5 months Apc\(^{\text{Min/+}}\) mice spontaneously develop numerous polyps throughout the small intestine. Rare polyps were detected in the colon, with no preferential distribution. In certain cases mice had splenomegaly and in rare cases females had also mammary tumours (Figure 3.1).

\[\text{Figure 3.1: Disease phenotype in } Apc^{\text{Min/+}} \text{ mice. (a) Small intestine of 5-month-old } Apc^{\text{Min/+}} \text{ mouse, divided into distal, medial and proximal portions (upper figure). Two representative colons from 5-month-old } Apc^{\text{Min/+}} \text{ mice (lower figure). The white bar corresponds to 5 mm in length. (b) Spleen from } Apc^{\text{Min/+}} \text{ mouse and WT healthy mouse. (c) Mammary tumour in } Apc^{\text{Min/+}} \text{ female.}]

In 5-month-old Apc\(^{\text{Min/+}}\) mice, different stages of tumour progression were detectable in tumour-bearing colons stained with H&E, from single aberrant crypts through aberrant crypt foci to adenomas, to eventually carcinomas. In the inset of the carcinoma, the submucosa was characterized by a massive infiltration of inflammatory cells (Figure 3.2a).

The transversal histological section of a WT healthy colon displayed regular crypts constituted by enterocytes and goblet cells, surrounded by the stromal components of the LP (Figure 3.2b, left). In contrast, in the two representative colon adenomas the well-ordered crypt architecture was replaced by aberrant iper-proliferative crypts and loss of goblet cells (Figure 3.2b, right). Hence, in Apc\(^{\text{Min/+}}\) mice the colonic epithelium was subjected to a progressive transition, from healthy to tumoural, in which the steady state homeostatic functions were lost and the abnormal proliferation was favoured.
Figure 3.2: Characterization of the tumour progression in Apc$^{Min/+}$ mice. (a) H&E staining of 4 μm-formalin fixed and paraffin embedded sections. From the left, four stages of development, i.e. single aberrant crypt, aberrant crypt focus, adenoma, carcinoma. From the left, light blue arrows indicate the aberrant crypt, the aberrant crypt focus and inflammatory cells. (b) Comparison between healthy colon (left) and adenoma (right).

3.1.2 Dextran Sodium Sulfate treatment accelerates the early onset of colonic tumours in Apc$^{Min/+}$ mice

Colorectal cancer formation can be experimentally accelerated by combining the genetic predisposition of Apc$^{Min/+}$ mice and a chemically-induced colitis. Based on the published literature (306), we administered 1-week treatment of 1.5% (w/v) DSS dissolved in autoclaved water to 8-week-old Apc$^{Min/+}$ male and female mice. At day 8, the DSS was replaced with drinking water. The body weight was measured at day 4 and daily from day 6 till the termination of the acute inflammation (day 13-14). The mice started losing body weight at day 6-7 and reached the maximum weight loss at day 10 showing signs of colitis such as blood in the stools, dehydration and diarrhea. The recovery phase from the acute colitis terminated at day 13-14.

Since the intensity of inflammation determines the tumour load, we aimed to induce in all our Apc$^{Min/+}$ mice a similar severity of colitis, characterized by a 10% body weight reduction, which allows to analyze an “unbiased” tumour burden at day 35 (day of harvesting and analysis) (Figure 3.3a). One-week cycle of 1.5% (w/v) DSS was demonstrated to be the optimal dosage to induce a colitis that was resolved by day 14, and triggered the onset of tumorigenesis. In contrast, 3% DSS induced a persisting acute colitis that fueled an excessive polyp burden in Apc$^{Min/+}$ mice (data not shown).

Similar to the spontaneous Apc$^{Min/+}$ mouse model, all stages of the tumour progression were detected in DSS-treated Apc$^{Min/+}$ mice, nevertheless the polyps were localized not in the small intestine but mostly in the large intestine, anatomical site affected by the
Results

DSS treatment. In fact, the tumours were preferentially localized in the distal and medial portions of the colon (Figure 3.3b) and occasionally the tumour vasculature was macroscopically visible (Figure 3.3c).

**Figure 3.3**: DSS accelerates tumour onset in *Apc<sup>Min/+</sup>* mice. (a) Schematic representation of the experimental protocol used to accelerate colon cancer development in *Apc<sup>Min/+</sup>* mice. (b) Representative colon of *Apc<sup>Min/+</sup>* mouse at day 35 post-start of DSS treatment. (c) Distal colon with two polyps visibly vascularized. The white bar represents a length of 3 mm.

Interestingly, advanced adenomas displayed an intrinsic heterogeneity, with at least two distinct areas of tumour progression and aggressiveness. Immunohistochemistry staining revealed higher protein levels of β-catenin in the apical part of the polyps, as opposed to higher protein levels of epithelial cell adhesion protein (EpCAM) in the basal part as well as in the tumour-surrounding epithelium (Figure 3.4a).

**Figure 3.4**: Tumour heterogeneity in colon polyps of *Apc<sup>Min/+</sup>* mice. (a) Immunohistochemistry staining in 9 μm-cryosections. From the left, β-catenin staining, EpCAM staining,
merging of both pictures. The white bar represents a length of 100 μm. (b) Comparison between healthy colon (upper figure) and adenoma (lower figure) in the expression levels of pan-cytokeratin. From the left, DAPI staining for the nuclei, pan-cytokeratin staining, merging of both pictures. The white bar represents a length of 100 μm. The red arrow points a tumour area with overexpression of the indicated marker. The dotted line separates two areas that display differential protein levels of the indicated marker. (Abbreviations: pan-cyto for pan-cytokeratin).

In contrast with the basal part, the apical part was characterized by tumoural epithelial cells with an aberrant accumulation of β-catenin, which is a downstream component of the Wnt/β-catenin pathway. EpCAM and pan-cytokeratin proteins were comparable in their expression levels. Similar to EpCAM staining, pan-cytokeratin had higher expression levels in both the apical portion of the crypts in normal colon as well as in the basal part of the adenomas (Figure 3.4b).

3.1.3 Gating strategy and definition of distinct cell subpopulations

As a next step, we aimed to characterize the intratumoural myeloid cell and lymphoid cell subsets at different stages of tumour progression using flow cytometry. Table 5 shows the used markers to delineate distinct immune cell subsets in normal colon LP.

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<tr>
<th>Cell subset</th>
<th>Abbreviation</th>
<th>Phenotype</th>
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<tr>
<td>Neutrophils</td>
<td>Neu</td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt;Ly6G&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Tissue-resident MHCII&lt;sup&gt;+&lt;/sup&gt; macrophages</td>
<td>MHCII&lt;sup&gt;+&lt;/sup&gt; or hi</td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt;F4/80&lt;sup&gt;+&lt;/sup&gt;MHCII&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Tissue-resident MHCII&lt;sup&gt;+&lt;/sup&gt; macrophages</td>
<td>MHCII&lt;sup&gt;-&lt;/sup&gt; or low</td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt;F4/80&lt;sup&gt;-&lt;/sup&gt;MHCII&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>Monocytes – population 1</td>
<td>P1</td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt;F4/80&lt;sup&gt;+&lt;/sup&gt;Ly6C&lt;sup&gt;-&lt;/sup&gt;MHCII&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>P2</td>
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<td>Monocyte-derived macrophages – population 3</td>
<td>P3</td>
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<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Regulatory T cells</td>
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<td>-</td>
<td>CD45&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt;B220&lt;sup&gt;+&lt;/sup&gt;</td>
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Table 5: Cell markers. Myeloid cell subsets and lymphoid cell subsets characterized by flow cytometry based on the differential expression of extracellular and intracellular markers. The abbreviation is frequently used to label the corresponding gated cell population in the representative plots. (Abbreviation: Fr: fraction).

Using a progressive gating strategy, neutrophils (neu; CD11b<sup>+</sup>Ly6G<sup>+</sup> cells), tissue-resident macrophages (low and hi; CD11b<sup>+</sup>F4/80<sup>-</sup>MHCII<sup>-</sup> and CD11b<sup>+</sup>F4/80<sup>+</sup>MHCII<sup>+</sup>)
cells), eosinophils (eos; CD11b+Ly6C+MHCII+ cells), monocytes (P1; CD11b+F4/80intLy6C+MHCII+ cells) and two subpopulations of monocyte-derived macrophages (P2 and P3; CD11b+F4/80intLy6C+MHCII+ and CD11b+F4/80intLy6C-MHCII+ cells) were detected not only in the LP (Figure 3.5, upper panel) but also in the intestinal tumours (Figure 3.5, lower panel), although with different frequencies (Figure 3.8 and described in section 3.1.4).

**Figure 3.5:** Gating strategy applied to visualize myeloid cell subsets in colon LP and tumour. CD45+ leukocytes are gated whereas CD45- cells are excluded from the analysis. Staining for CD11c and MHCII markers enables to exclude CD11c-MHCII- dendritic cells. The non-dendritic cell population contains CD11b-Ly6G- neutrophils and two CD11bhi cell subsets with different expression levels of F4/80: F4/80int tissue-resident macrophages (fraction I) and F4/80int myeloid cell subset (fraction II). F4/80int macrophages can be further subdivided into F4/80intMHCIIlo and F4/80intMHCIIhi fractions (abbreviated as low and hi, respectively). F4/80int gate is a mixture of 4 cell populations: eosinophils (eos), monocytes (P1) and two monocyte-derived Ly6C+ and Ly6C- macrophage subsets (P2 and P3). Upper panel: LP; lower panel: tumour.

In order to visualize their morphology, distinct myeloid cell subsets were purified via cell sorting from the formed colon tumours and stained with Giemsa (Figure 3.6). Clear
macrophage morphology could be detected in P1-P3 as well as in both MHCII\textsuperscript{lo} and MHCII\textsuperscript{hi} cells, whereas the neutrophil subset contained cells with both hyper-segmented and ring-shaped nuclei, which indicates the presence of mature neutrophils and MDSCs in this fraction.

![Figure 3.6: Morphology of sorted intratumoural myeloid subsets.](image)

From the left, representative picture of P1 cells (monocytes); P2 cells (Ly6C\textsuperscript{+}MHCII\textsuperscript{+} macrophages); P3 cells (Ly6C MHCII\textsuperscript{+} macrophages); F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} and F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} tissue-resident macrophages; a mixed cell population of hyper-segmented and mature neutrophils and immature cells with a ring-shaped nucleus, likely granulocytic MDSCs; eosinophils. The black bar represents a length of 10 μm.

By applying the gating strategy in Figure 3.7, B cells (CD3\textsuperscript{-}B220\textsuperscript{+} cells), CD8\textsuperscript{+} T cells (CD3\textsuperscript{-}CD8\textsuperscript{-} cells) CD4\textsuperscript{+} T cells (CD3\textsuperscript{-}CD4\textsuperscript{-} cells), Tregs (CD3\textsuperscript{-}CD4\textsuperscript{-}Foxp3\textsuperscript{+} cells) and γ/δ T cells (CD3\textsuperscript{-}CD4\textsuperscript{-}CD8\textsuperscript{-}γδTCR\textsuperscript{+} cells) were identified in both LP (upper panel) and tumours (lower panel), even though markedly different in their frequencies (Figure 3.8).

![Figure 3.7: Gating strategy applied to visualize lymphoid cell subsets in colon LP and tumour.](image)
cells. The latter cell population contains Foxp3+ Tregs. A percentage of CD3+CD4+CD8- cells are γ/δ T cells. From the CD45+ cell compartment, CD3- B220+ B cells are gated. Upper panel: LP; lower panel: tumour.

The colonic LP (4 weeks post-DSS treatment) contained predominantly B cells, which made up 70% of the leukocytes, whereas the myeloid cells were 10% of the total leukocytes (Figure 3.8, left pie). In contrast, DSS-accelerated tumours were poorly infiltrated by B cells (10% of the leukocytes) and displayed a higher proportion of myeloid cells (at least 48% of the leukocytes), particularly neutrophils. (Figure 3.8, right pie). As a side note, a fraction of CD45+ cells in LP and tumours has not been characterized, and corresponded to 11% and 30% respectively. This fraction may include DCs, Mast cells, NK cells as well as NK T cells.

![Figure 3.8: Lymphoid and myeloid cell heterogeneity in colon LP and colon tumours. Pies showing the abundance of myeloid and lymphoid cell populations obtained from DSS-treated LP 4 weeks post-treatment (n=10) (left pie) and 2-3 mm DSS-accelerated colon tumours (n=10) (right pie), expressed as percentage of CD45+ cells. Light grey section: percentage of non-characterized CD45+ cells.](image)

**3.1.4 Myeloid cell heterogeneity in LP and tumour**

Using the flow cytometry strategy previously described, a detailed comparative analysis of myeloid cell subsets, expressed as percentage of CD11b+ myeloid cells, was performed in the DSS-treated LP and in DSS-accelerated tumours 4 weeks post-DSS treatment (Figure 3.9).

In the colon LP, the majority of tissue-resident macrophages (fraction I) were MHCII\textsuperscript{hi} macrophages, whereas a sparse percentage was MHCII\textsuperscript{lo}. Within F4/80\textsuperscript{int} myeloid cells (fraction II), the most abundant cell subpopulation was represented by the eosinophils, followed by monocytes (P1), and monocyte-derived macrophages (P2-P3). All the LP myeloid cell populations were identified in DSS-accelerated colon tumours as well, although with markedly different frequencies. Indeed, neutrophils (fraction III) were the most abundant myeloid cell type (more than 40% of CD11b+ myeloid cells) and monocytes increased from 1.5% to 6%. As opposed to the LP, in the tumour the
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eosinophils were a minor fraction (about 3% of CD11b+ myeloid cells). Furthermore, tumour-resident macrophages (fraction I) were clearly enriched in the MHCIIlo cell fraction, which was almost negligible in the adult colon LP.

![Diagram](attachment:image.png)

**Figure 3.9: Myeloid cell heterogeneity in colon LP and colon tumours.** (a) 8-week-old WT and ApcMin/+ mice were given 1-week DSS treatment and analysed 4 weeks later. (b) Flow cytometry representative dot plots of colon LP (upper panel) and tumour cell subpopulations (lower panel). After pre-gating on CD45+ cells and excluding dendritic cells, Fraction I-III are defined by the differential expression of F4/80. Fraction I represents F4/80hi tissue-resident macrophages, further subdivided into MHCIIhi and MHCIIlo subpopulations. Fraction II contains monocytes (P1), two monocyte-derived macrophage subpopulations (P2 and P3) and eosinophils (eos). Fraction III consists of neutrophils. (c) Bar charts showing the abundance of the analysed myeloid cell populations from the colon LP (n=8) (upper bar chart) and 2-3 mm colon tumours (n=20) (lower bar chart). White bars: fraction I; light blue bars: fraction II and dark blue bars: fraction III. Error bars represent the s.e.m. (d) Pie charts showing the proportions of the same myeloid cell subsets in Figure 3.9c, in colon LP (upper pie chart) and tumours (lower pie chart).

The characterization of spontaneously developed colon tumours in 6-month-old ApcMin/+ mice confirmed the presence of all myeloid subpopulations described in Figure 3.9, although a clear increase in the proportion of intratumoural neutrophils (up to 70% of CD11b+ myeloid cells) and decrease of tissue-resident macrophages were observed (Figure 3.10). In this case, the colon of ApcMin/+ mice was not subjected to the DSS-
induced acute colitis and the subsequent infiltration of proinflammatory cells such as monocytes, which ultimately differentiate into macrophages. Hence, a physiological low-grade inflammation could explain the reduced proportion of tissue-resident macrophages, when compared to that one in DSS-accelerated tumours.

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**Figure 3.10: Myeloid cell heterogeneity in colon WT LP and spontaneous colon tumours.**
(a) The abundance of myeloid cell populations was analysed in colon tumours spontaneously developed in 6-month-old ApcMin/+ mice and was compared to that one of age-matched WT untreated mice. (b) Flow cytometry representative dot plots of colon LP (upper panel) and tumour cell subpopulations (lower panel). The same gating strategy is described in figure 3.9b. (c) Bar charts of the distinct myeloid cell subpopulations obtained from the colon LP (n=7) (upper bar chart) and 2-3 mm colon tumours (n=8) (lower bar chart). White bars: fraction I; light blue bars: fraction II and dark blue bars: fraction III. Error bars represent the s.e.m. (d) Pie charts showing the proportions of the analysed myeloid cell subsets in colon LP (upper pie chart) and tumour (lower pie chart).

Of note, in both experimental tumour models neutrophils were the predominant infiltrating leukocyte population. To understand whether the neutrophil accumulation was an early or late event in the tumour progression, spontaneously induced tumours of various sizes were collected and analyzed (Figure 3.11). Regardless of the size, neutrophils represented about 45-60% of the CD45+ cells in the tumour, being the most abundant myeloid cells as early as the first stages of the tumour development.
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Figure 3.11: Massive infiltration of neutrophils is an early-occurring event. From the left, representative pictures of healthy colon LP in WT mice, and spontaneously induced tumours of 1 mm, 2 mm, 4 mm and 6 mm of diameter in Apc<sup>Min/+</sup> mice, and the corresponding CD11b-Ly6G flow cytometry plots. In the plot, CD11b+Ly6G+ neutrophils were gated. The white bar represents a length of 4 mm. Bar chart of the neutrophil numbers, expressed in percentage of CD45<sup>+</sup> cells. LP: n= 4; 0.5 - 1 mm: n=3; 2 - 3 mm: n=2; 4 mm: n=2; 5 - 6 mm: n=2. The error bars represent the s.e.m. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. **P<0.01; ***P < 0.001.

3.1.5 Modifications in the tumour microenvironment during tumour progression

A growing body of evidence has shown that the tumour microenvironment hosts a unique composition of stromal cells, including myeloid and lymphoid cells (Figure 3.8,131,132,316). With time, the dynamic interaction between stromal and neoplastic cells changes in favour of tumour progression, by promoting tumour cell proliferation, survival, and metastasis as well as by shaping an immunosuppressive environment (131,132). Thus, we characterized the abundance of cytokines and factors at different stages of tumour development and compared it with the LP milieu (Figure 3.12).

In order to obtain tumours of different sizes up to 5-6 mm in diameter, spontaneous tumours from 6-month-old Apc<sup>Min/+</sup> mice and the LP from age-matched WT mice were processed for total RNA isolation. The cDNA levels of factors involved in ECM remodelling (Mmp12, Mmp9), tumour angiogenesis (Hif1α, Angpt2, Vegfa), recruitment and survival of myeloid cells (Ccl2, Csf1, Csf2, Csf3) and macrophage polarization towards M2-like phenotype (Arg1, Marco) were significantly enriched in the advanced tumoural stages. The kinetics of accumulation and the abundance varied according to the tested factor. On the contrary, Ifny (produced by activated CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells (317)) progressively diminished during the tumour progression.
Figure 3.12: Quantification of cytokines and factors in the tumour microenvironment at different stages of the tumour progression. (a) The abundance of factors/cytokines in colon tumours spontaneously developed in 6-month-old ApcMin/+ mice was compared to that one in LP obtained from age-matched WT untreated mice. (b) Intratumoural Arg1, Marco, Mmp9, Mmp12, Hif1α, Angpt2, Vegfa, Ccl2, Csf1, Csf2, Csf3 RNA levels gradually increase with tumour progression and exhibit different kinetics of accumulation and fold-change. On the contrary, intratumoural Ifnγ does not vary in its expression. Colon LP and polyps of different sizes (ranging from 0.5 mm to 6 mm) from 6-month-old ApcMin/+ mice were processed for quantitative PCR analysis of expression. For each target, data were normalized to β-actin and were expressed as fold-change in gene expression from the sample showing the lowest expression. LP: n=6; 0.5 mm: n=2; 1-1.5 mm: n=4; 2 mm: n=4; 3-4 mm: n=8; 5-6 mm: n=8. Error bars represent the s.e.m. (Abbreviations: Arg1: Arginase 1; Mmp: Matrix metalloproteinase; Hif1α: Hypoxia-inducible factor 1-alpha; Angpt2: Angiopoietin 2; Vegfa: Vascular endothelial growth factor A; Ccl2: C-C motif chemokine ligand 2; Csf: Colony-stimulating factor; Ifnγ: Interferon-gamma).

3.1.6 MHCII+ macrophages disappear at the onset of the adulthood

After observing that neutrophils were the most abundant intratumoural myeloid cell population, and massively infiltrated the tumour immediately at the earliest tumoural stage, we also assessed the kinetics of infiltration and eventually accumulation of different subsets of F4/80hi tissue-resident macrophages (high and low). Before analysing the tumours, we compared fraction I in the fetal colon (E19.5) with that one in colon LP of young and old mice (from 1 week to 12 months of age).
Figure 3.13: Ageing influences the ratio between F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>lo</sup>MHCII<sup>lo</sup> subpopulations. (a) All myeloid cells analysed, including F4/80<sup>hi</sup> tissue-resident macrophages, persist in the colon LP after birth into adulthood, as shown by the representative flow cytometry dot plots and bar chart. Error bars represent the s.e.m. White bars: fraction I; light blue bars: fraction II and dark blue bars: fraction III. (b) The frequency of colon LP F4/80<sup>hi</sup>MHCII<sup>lo</sup> cells rapidly declines after birth. Representative dot plots of F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> subpopulations obtained from fetal colon (E19.5) and from colons of mice aged 2 days, 1, 3, 4, 6, and 8 weeks and 12 months. The bar chart represents the age-dependent ratio between F4/80<sup>hi</sup>MHCII<sup>hi</sup> (blue) and F4/80<sup>hi</sup>MHCII<sup>lo</sup> (red) subpopulations in the colon LP. E19.5: pool of 15 colons of embryos obtained from 3 different pregnant mice; day 2: n= 2 (in each sample 5 colons were pooled); 1 week: n= 2 (in each sample 5 colons were pooled); 3 weeks: n= 6 mice; 4 weeks: n= 5 mice; 6 weeks: n=4; 8 weeks: n= 7 and 12 months: n= 4 mice. Error bars represent the s.e.m. Gating strategy is shown in Figure 3.5.
Fractions I–III were present at all timepoints of age with major fluctuations in numbers from E19.5 to the first week of life, most likely due to the first exposure of the gut to a different environment, compared to that one in the placenta, and to the colonization with commensals. Of note, 2 days after birth the neutrophils massively infiltrated the colon LP, to decline again in numbers within one week of age. Tissue-resident macrophages (fraction I) dramatically accumulated in the LP of 1-week-old mice, becoming the most abundant myeloid cell population. From the third week after birth minor fluctuations in the frequencies were detected. These data suggested that the cell composition of the immune system in the intestinal LP was shaped in the first days/first week after birth (Figure 3.13a).

The predominant tissue-resident macrophage subset in the fetal colon was the F4/80hiMHCIIlo fraction. However, as a function of age, this macrophage subset almost disappeared to leave mainly F4/80hiMHCIIhi cells to represent the colon tissue-resident macrophages (fraction I) (Figure 3.13b). The decrease in MHCIIlo macrophages was likely related to the process of ageing, which is characterized by the establishment of intestinal microbiota and a tonic increase of inflammatory mediators (59).

3.1.7 Influence of the microbiota on the colonic myeloid cell compartment

To assess whether the microbiota could influence the frequency of the myeloid cell subsets, we performed an experiment in germ free (GF) mice. The LP myeloid cell composition in 8-week-old GF mice was compared to that one in 8-week-old conventionally-housed mice (CV) (Figure 3.14). Clearly, GF colon LP had significantly lower infiltration of neutrophils and monocytes, whereas the observed differences in cell numbers of P2-P3 subsets, DCs as well as MHCIIlo and MHCIIhi macrophages were statistically non-significant. More GF mice should be analysed in the future to solidify these data. As already reported, GF mice carried a visibly enlarged caecum (318) (Figure 3.14d).
Results

Figure 3.14: Gut microbiota influences the abundance of monocytes and neutrophils in steady state colon LP. (a) All LP myeloid cell subsets in CV mice were also found in GF mice, as shown by the representative flow cytometry dot plots. (b) Bar charts show the abundance of each myeloid cell subset, expressed in absolute numbers (left panel) and in percentage of CD45+ cells (right panel). Error bars represent the s.e.m. Statistical significance was determined using an unpaired Student's t-test. *P<0.05; **P < 0.001; ns: not significant. (c) Pie charts show the proportions of all myeloid cell subpopulations analysed across colon LP in CV mice (left pie chart) and GF mice (right pie chart). Gating strategy is shown in Figure 3.5. (d) Two examples of enlarged caecum from GF mice, compared to normal caecum from steady state WT mice (Abbreviations: CV: conventionally-housed; GF: germ free; DCs: dendritic cells).

3.1.8 MHCIIlo macrophages accumulate during tumour progression

Given the different frequencies of F4/80lo MHCIIlo macrophages in colon tumours and LP, respectively (Figure 3.9), we assessed whether there was an association between tumour progression and the presence of intratumoural MHCIIlo macrophages. We analysed DSS-treated ApcMin/+ mice and subdivided the tumours into three categories: polyps with diameter smaller than 2 mm, in the range of 2-3 mm and bigger than 3 mm.
While the abundance of neutrophils, monocytes, P2-P3 macrophages, eosinophils and MHCII<sup>lo</sup> resident-macrophages was maintained during tumour progression, the MHCII<sup>lo</sup> macrophages significantly and gradually increased in their proportion (Figure 3.15a-b). To perform a more extensive characterization we subsequently opted for the spontaneous tumour model of Apc<sup>Min/+</sup> mice, since they progressively generated adenomas up to 5 mm in size that were rarely achieved in the DSS-accelerated tumour model. Again, we observed an increasing abundance of the MHCII<sup>lo</sup> macrophage fraction while the tumour progressed. In large tumours of 5-6 mm in diameter, MHCII<sup>lo</sup> macrophages became the predominant tumour-resident F4/80<sup>hi</sup> macrophage subpopulation (Figure 3.15c).

![Figure 3.15: Tumour progression influences the ratio between F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>lo</sup>MHCII<sup>lo</sup> subpopulations.](image)

(a) Representative flow cytometry dot plots of fractions I-III in DSS-accelerated tumours with diameter less than 2 mm, between 2-3 mm and more than 3 mm.
(upper panel). In the lower panel, dot plots with the proportions of MHCII<sup>lo</sup> and MHCII<sup>hi</sup> macrophages from the corresponding fraction I. (b) Bar charts summarizing the abundance of intratumoural myeloid cell subsets, expressed in percentage of CD11b<sup>+</sup> myeloid cells, from DSS-accelerated tumours and in three different categories of tumour diameter (i.e. diameter < 2 mm, 2-3 mm, > 3 mm). < 2 mm: n= 14; 2-3 mm: n= 13; > 3 mm: n= 7. Error bars represent the s.e.m. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. *P<0.05, **P<0.001. Non-significant difference is not shown. (c) Representative flow cytometry analysis (left panel) and bar chart with the ratio F4/80<sup>hi</sup>MHCII<sup>hi</sup>:F4/80<sup>hi</sup>MHCII<sup>lo</sup> subpopulations (right panel) in tumours of different sizes (0.5-6.0 mm) obtained from Apc<sup>Min+</sup> mice. 0.5 mm: n= 3; 1 mm: n= 3; 2mm: n= 4; 3-4 mm: n= 3; 5-6 mm: n= 4. Error bars represent the s.e.m.

### 3.2 Macrophage turnover and maintenance

#### 3.2.1 Tumour-resident macrophages are not derived from CCR2-dependent monocytes

Given that circulating Ly6C<sup>hi</sup> monocytes express high levels of CCR2, which is the most important chemokine receptor involved in the recruitment of circulating monocytes from the BM to the colon LP (59), we examined by flow cytometry the expression levels of this receptor on tissue-resident and monocyte-derived macrophages in LP and adenomas. In the LP, consistent with being progenies of classical monocytes, Ly6C<sup>hi</sup>MHCII<sup>hi</sup> (P1), Ly6C<sup>hi</sup>MHCII<sup>lo</sup> (P2) and Ly6C<sup>lo</sup>MHCII<sup>hi</sup> (P3) cells had the highest and most homogeneous expression of CCR2, whereas tissue-resident F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>lo</sup>MHCII<sup>lo</sup> macrophages were highly heterogeneous in their CCR2 expression levels. Overall, in the LP MHCII<sup>hi</sup> macrophages had a higher percentage of CCR2-expressing cells and a higher CCR2 expression level compared to MHCII<sup>lo</sup> macrophages. Interestingly, the majority of tumour-resident macrophages were lacking this chemokine receptor. Only a minor fraction of intratumoural F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>lo</sup>MHCII<sup>lo</sup> macrophages expressed CCR2 and definitively at lower levels when compared to the LP counterparts (Figure 3.16b-c).

As a next step, RNAseq analysis was performed to determine the expression of other receptors known to be involved in macrophage recruitment and/or survival/proliferation. Four cell subsets (MHCII<sup>hi</sup> and MHCII<sup>lo</sup> tissue-resident macrophages, monocytes and neutrophils) from both tumour and LP were sorted based on the surface markers and gating strategy in Figure 3.17b. Each sorted cell subset had high purity and reached sufficient cell numbers for the RNA isolation and subsequent RNAseq. Based on the RNAseq heat map, both tumour-resident macrophage subsets increased their expression levels of Cx3cr1 and Csf1r, which were lower in the LP counterparts. Ccr5 was more expressed in LP MHCII<sup>hi</sup> macrophages compared to LP MHCII<sup>lo</sup> macrophages, and this trend was maintained in the tumour, suggesting no major changes in Ccr5 expression levels between healthy and malignant tissues. Both Csf2r and Csf3r, crucial for neutrophil recruitment, had a low expression in both LP and
intratumoural macrophage subsets. Ccr7, expressed in LP MHCII$^{hi}$ macrophages, was downregulated in the tumour counterparts. Overall, tumour-resident MHCII$^{hi}$ macrophages downregulated Ccr2 and Ccr7, upregulated Cx3cr1 and Csf1r and preserved the expression profile of Ccr5. Tumour-resident MHCII$^{lo}$ upregulated Cx3cr1 and Csf1r and preserved the expression profile of Ccr5. Tumour-resident MHCII$^{lo}$ upregulated Cx3cr1 and Csf1r. These data suggest that in the tumour environment of colon adenomas tissue-resident macrophages required Cx3cr1, Csf1r and Ccr5 for their maintenance, whereas Ccr2 and Ccr7 became dispensable similarly to Csf2r and Csf3r (Figure 3.17c).

Figure 3.16: LP and intratumoural F4/80$^{hi}$ macrophage subsets show differential CCR2-expression. (a) 8-week-old WT and Apc$^{Min/+}$ mice were administered DSS for 7 days. Colon tumours and DSS-treated LP were analysed 4 weeks later. (b) Representative histograms (upper panel) and representative flow cytometry plots (lower panel) showing extracellular CCR2 expression on distinct colon LP and intratumoural myeloid cell subpopulations: fraction I consists
of MHCII<sup>lo</sup> and MHCII<sup>hi</sup> macrophages and fraction II consists of eosinophils, monocytes (P1) and monocyte-derived macrophages (P2 and P3). (c) Bar charts representing CCR2 expression levels (Mean Fluorescence Intensity (MFI), right lower panel) and percentage of CCR2-expressing cells (left lower panel). Black bars: LP (n=6) and white bars: tumour (n=6). Error bars represent the s.e.m. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. *P<0.05; **P < 0.001; ns: not significant.

Figure 3.17: Differential expression of Ccr2, Ccr7, Cx3cr1 and Csfr between LP and intratumoural F4/80<sup>hi</sup> macrophages. (a) To obtained MHCII<sup>lo</sup>, MHCII<sup>hi</sup>, P1 monocytes and neutrophils from LP and tumour, the sorting was performed 4 weeks after DSS treatment in WT and Apc<sup>Min/+</sup> mice respectively. (b) Representative gating strategy of tumour sample and plots (bottom panel) showing the highly pure cell populations achieved with the sorting. The same gating strategy was used for LP samples. (c) Heat map of differentially expressed genes (DEGs) between LP monocytes, neutrophils, MHCII<sup>lo</sup> and MHCII<sup>hi</sup> macrophages, and their cell counterparts in the tumour. A triplicate of each sorted cell population is reported. Each value of the triplicate corresponds to a biological replicate that was obtained by pooling about 10-15 mice both for tumour and LP. The heat map was generated with log2 transformed RPKM values and
with the row/gene median subtracted. Both the row and columns were clustered using hierarchical clustering with Euclidean distance and complete linkage method.

Similarly to the extracellular CCR2 expression (Figure 3.16b-c) we defined the surface protein levels of CX3CR1 on LP and intratumoural tissue-resident MHCII\(^\text{lo}\) and MHCII\(^\text{hi}\) macrophages, monocytes and neutrophils by utilizing Cx3cr1\(-\text{gfp}\) mice and Cx3cr1\(-\text{gfp}\ Apc^{\text{Min}+}\) mice, which carry a transgene containing the coding sequence of Cx3cr1 linked to the coding sequence of Green fluorescence protein (Gfp). GFP labelling occurs in those cells expressing the transgene Cx3cr1 (Figure 3.18).

![Diagram](image)

Figure 3.18: Both intratumoural F4/80\(^\text{hi}\) macrophage subsets display a high expression of CX3CR1. (a) Cx3cr1\(-\text{gfp}\) mice and Cx3cr1\(-\text{gfp}\ Apc^{\text{Min}+}\) mice were administered 1-week DSS treatment. Colon LP and tumours were analysed after 4 weeks. (b) Using the flow cytometry gating strategy in Figure 3.5, representative histograms show the CX3CR1 expression in each cell subset analysed (MHCII\(^\text{lo}\) and MHCII\(^\text{hi}\) macrophages, P1 monocytes and neutrophils) from LP (n=3) and tumour (n=3).

Both tumour-resident macrophage subsets were CX3CR1\(^+\) and carried high surface levels of this receptor, whereas all monocytes had an intermediate expression and neutrophils were CX3CR1\(^-\). In the LP, MHCII\(^\text{lo}\) macrophages appeared to be a heterogeneous cell population with regards to the surface CX3CR1 expression, since there were clearly two fractions expressing or lacking this chemokine receptor. The LP MHCII\(^\text{hi}\) macrophages, P1 and neutrophils had overlapping expression profiles with the corresponding intratumoural counterparts.

Taken together, the tumour microenvironment altered the expression levels of certain chemokine/cytokine receptors in tissue-resident macrophages. Most remarkably, intratumoural F4/80\(^\text{hi}\)MHCII\(^\text{hi}\) macrophages were CCR2\(^{-}\), similarly to MHCII\(^\text{lo}\) macrophages in both LP and tumour. To further define the CCR2-dependency for each myeloid cell subset and understand the downstream effects of the absence of CCR2, we quantified the myeloid cells in LP and tumour obtained from Ccr2\(^{\text{lox/lox}}\) mice and Ccr2\(^{\text{lox/lox}}\).
Apom+° mice, respectively. As expected, in the LP of Ccr2−/− mice, monocytes and monocyte-derived cells (P1, P2 and P3) were severely impaired in absolute numbers. In addition, as already reported (59), MHCII hi tissue-resident macrophages were dramatically reduced due to their CCR2-dependency. On the other hand, MHCII lo macrophages were not affected in numbers, which is likely a reflection of their markedly lower CCR2 expression level and CCR2-independency (Figure 3.19b). Interestingly, the absolute numbers of tumour-resident MHCII hi and MHCII lo macrophages were not reduced in Ccr2−/− mice. On the contrary, intratumoural P1, P2 and P3 cells were similarly affected as their LP counterparts (Figure 3.19c).

Figure 3.19: LP and intratumoural F4/80 hi macrophage subsets show differential CCR2-driven monocyte dependence. (a) 8-week-old WT, Ccr2−/−, Ccr2−/− Apom+° and Apom+° mice were administered DSS for 7 days. Colon tumours and DSS-treated LP were harvested and analysed 4 weeks later. (b) Myeloid cell profiling in WT and Ccr2−/− colon LP. Representative flow cytometry plots of F4/80 and CD11b-expressing myeloid subpopulations, obtained from the colon LP of WT and Ccr2−/− mice. Fractions I and II were further dissected based on MHCII and Ly6C expression (left panel). The absolute numbers of eosinophils, F4/80 hi MHCII hi and F4/80 hi MHCII lo tissue-resident macrophages and P1-P3 subpopulations are shown (right panel). In the bar charts, the bar represents the mean of the absolute numbers of each myeloid cell subset and the error bar represents the s.e.m. (WT: n=7 and Ccr2−/−: n=14). (c) Myeloid cell
profiling in WT and Ccr2−/− colon tumours. Bar charts show the mean ± s.e.m. of absolute numbers of eosinophils, F4/80hiMHCIIhi and F4/80hiMHCIIlo tissue-resident macrophages and P1-P3 subpopulations obtained from ApcMin/+ (n=8) and Ccr2−/−ApcMin/+ (n=9) mice. Statistical significance was determined with unpaired Student’s t-test. *P < 0.05; **P < 0.001; ***P < 0.0001; ns: not significant. Gating strategy is shown in Figure 3.5.

3.2.2 The maintenance of LP-resident and tumour-resident macrophages does not rely on CSF2R-CSF axis

Since alveolar macrophages depend on GM-CSF (also known as CSF2) (49), we investigated whether intestinal macrophages require CSF2R-CSF axis to maintain their numbers. We dissected the myeloid cell compartments in the LP of Csf2r−/− mice as well as in the tumour of Csf2r+/−ApcMin/+ mice and compared them with the counterparts in the controls, Csf2r+/+ and Csf2r+/+ ApcMin/+ mice (Figure 3.20).

![Figure 3.20: CSF2R-CSF2 axis is indispensable for eosinophil recruitment in the intestine.](image)

(a) 8-week-old Csf2r+/+, Csf2r−/−, Csf2r−/− ApcMin/+ and Csf2r+/+ ApcMin/+ mice were administered DSS for 7 days. Colon tumours and DSS-treated LP were harvested and analysed 4 weeks later. (b) Representative flow cytometry plots of myeloid cell subsets obtained from the colon LP of Csf2r+/+ and Csf2r−/− mice (left panel). The absolute numbers of neutrophils, eosinophils, F4/80hiMHCIIhi and F4/80hiMHCIIlo tissue-resident macrophages and P1-P3 F4/80int cell subpopulations are shown in the right panel. Bar charts represent the mean ± s.e.m. of the

Results
absolute numbers of each cell subset (Csf2r+/+: n=10 and Csf2r−/−: n=5). (c) Myeloid cell profiling in Csf2r+/+ and Csf2r−/− colon tumours. Bar charts indicate the mean ± s.e.m. of absolute numbers obtained from Csf2r+/+ ApcMin/+ (n=8) and Csf2r−/− ApcMin/+ (n=8) mice. Statistical significance was determined with unpaired Student’s t-test. *P < 0.05; ***P < 0.0001; ns: not significant. Gating strategy is reported in Figure 3.5.

The absence of CSF2R affected only the recruitment of eosinophils, which were severely impaired in their numbers in Csf2r−/− LP, and partially ablated in the tumours of Csf2r−/− ApcMin/+ mice, whereas the other myeloid cell subpopulations were comparable in numbers between WT and receptor-deficient mice.

In conclusion, in the tumour environment both tissue-resident macrophage fractions did not require CSF2R-CSF2 axis to preserve their cell pool and became independent from the CCR2-CCL2 axis, which was an indispensable axis of recruitment and refilling for LP tissue-resident F4/80hi MHCII hi macrophages.

3.2.3 Fate mapping to trace the cell ontogeny and turnover rate

In our laboratory, Sheng et al. (40) have generated a KitMerCreMer/R26 mouse strain that allows to fate map distinct cell types, including adult macrophages, at different timepoints of mouse development. This mouse strain carries a tamoxifen-inducible MerCreMer (Cre recombinase flanked by Murine estrogen receptor) construct that is inserted into the 3′-UTR of the Kit gene. Since Kit is expressed in early YS and FL progenitors as well as in fetal and adult HSCs and their early progenies, but not in mature hematopoietic cells (319), upon tamoxifen injection the constitutive YFP labelling occurs only in Kit+ progenitors, and consequently their progeny. KitMerCreMer/R26 mice can be used for embryonic as well as adult fate mapping.

![Figure 3.21: Embryonic and adult fate mapping.](image-url)

(a) Example of embryonic fate mapping with a single tamoxifen pulse at E10.5. Left panel: schematic illustration of E10.5 definitive...
haematopoiesis in the FL. Late EMPs, generated at E8.25, colonize the FL. At E8.5 HSCs are formed and subsequently seed the FL as well. Both progenitors give rise to fetal monocytes that differentiate into tissue-resident macrophages in all tissues, except microglia and partially Langerhans cells. Right panel: a single tamoxifen injection to pregnant mice carrying KitMerCreMer/R26 embryos at E10.5 permanently "turns on" the YFP signal in the Kit+ embryonic progenitors and descendants, excluding microglia and a fraction of Langerhans cells. (b) Left panel: schematic illustration of adult haematopoiesis in the BM. HSCs give rise to monocytes that differentiate into tissue resident macrophages in all tissues, except microglia and partially Langerhans cells. Right panel: a single tamoxifen injection to pregnant mice carrying KitMerCreMer/R26 embryos at E10.5 permanently "turns on" the YFP signal in the Kit+ embryonic progenitors and descendants, excluding microglia and a fraction of Langerhans cells. (Abbreviations: E: embryonic day; FL: fetal liver; BM: bone marrow; YFP: yellow fluorescence protein; EMP: erythromyeloid progenitor; HSC: hematopoietic stem cell).

When tamoxifen is injected as single dose during gestation, it is possible to tag the Kit+ precursors restricted to the chosen embryonic day. As an example, a tamoxifen pulse at E10.5 (Figure 3.21a) is expected to induce the YFP labelling of embryonic precursors (EMPs/HSCs) and their progenies, including fetal monocytes that give rise to the adult tissue-resident macrophages, except brain microglia and partially epidermal Langerhans cells since descendants of YS precursors (40).

When tamoxifen is administered as multiple injections in adult mice, followed by a chase period to induce the YFP labelling, only adult BM-HSCs and the progenies become labelled (Figure 3.21b). Those tissue-resident macrophages that are derived from earlier precursors cannot be tagged in adult mice. However, intestinal macrophages are constantly generated from the adult BM, hence become labelled similarly to all BM-derived cell populations, such as monocytes and neutrophils. (40)

The adult fate mapping system can also be used to define the turnover rate of BM-derived cell populations by modulating the chase period (Figure 3.22). Moreover, it is possible to assess whether a certain cell type alters its BM-dependency in response to a perturbed environment, for instance in cancer (Figure 3.24).

3.2.4 Turnover of myeloid cells in colon LP

In order to define whether the accumulation of tumour-resident macrophages was due to cell replenishment from the BM, we firstly defined the turnover rates of each myeloid cell population in the colon LP. We utilized the adult KitMerCreMer/R26 fate mapping system (Figure 3.21b), in which Yellow fluorescence protein (Yfp) expression can be induced in Kit+ BM progenitors. After administering tamoxifen to adult mice, the labelling index of tissue-resident macrophages (F4/80^hi MHCII^lo and F4/80^hi MHCII^hi), monocytes (P1), monocyte-derived macrophages (P2 and P3), eosinophils and neutrophils was determined at different timepoints over a period of 5 months (Figure 3.22a). Neutrophils in the LP were chosen as reference cell population since these cells are fully derived from the BM and remarkably short-lived with a circulating half-life of 6–8 hours. All
Results

tested myeloid cell populations, with the exception of tissue-resident macrophages, were fast YFP labelled and reached a plateau by 1–2 weeks after the last tamoxifen injection, suggesting that these myeloid cell populations were rapidly replaced by BM-derived cells.

Figure 3.22: Intestinal tissue-resident macrophages exhibit a slower, gradual replacement by BM-derived cells compared to other intestinal myeloid cells. (a) Schematic representation of the adult fate mapping protocol using KitMerCreMer/R26 mice. Eight-week-old mice were administered tamoxifen for 5 consecutive days and groups of 4–8 mice were sacrificed 1, 2, 3, 4, 7 and 20 weeks later. (b) Representative flow cytometry analysis indicating the labelling percentage of distinct colon myeloid cell populations: MHCIIlo and MHCIIhi tissue-resident

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macrophages, monocytes (P1) and monocytes-derived macrophages (P2-P3), eosinophils and
neutrophils. For each sample, neutrophils were considered as the internal control for normalizing
the labelling percentage of each cell subset. Gating strategy is shown in Figure 3.5. (c) The bar
chart represents the mean percentage of YFP+ cells after normalization to the percentage of
YFP+ neutrophils. The error bars represent the s.e.m.

Although it is widely demonstrated that in the adulthood intestinal F4/80hiMHCIIhi
macrophages lose their embryonic origin as they are progressively replaced by
monocyte-derived macrophages (58,59), this experiment provided further insight into
the turnover rate of each macrophage subset in the LP. F4/80hiMHCIIhi were clearly
refilled by BM-derived precursors, but with a slower rate compared to P2 and P3
fractions. At least 5 months were required to almost totally replace the resident
F4/80hiMHCIIhi cell subset (85% of YFP labelling); moreover, F4/80hiMHCIIlo
macrophages exhibited minimal labelling (20% at 5 months post-tamoxifen) (Figure
3.22b-c), suggesting that these sparse tissue-resident macrophages retained their
original macrophage population that seeded the fetal LP.

3.2.5 MHCIIlo macrophages in the LP are partially refilled by BM-derived macrophages

To provide evidence that F4/80hiMHCIIlo macrophages in the adult colon LP display the
lowest BM-dependency when compared to monocyte-derived macrophages (P2-P3)
and F4/80hiMHCIIhi resident macrophages, we generated BM chimeric mice. Recipient
congenic C57BL/6 Cd45.1+/Cd45.2+ mice were lethally irradiated and subsequently i.v.
injected with BM cells from Cd45.1+ donor mice. Ten weeks after reconstitution, the
colon LP was processed and analysed (Figure 3.23a).

![Image](image_url)

**Figure 3.23:** After BM irradiation, tissue-resident MHCIIlo macrophages are partially
derived from progenitors of reconstituted BM. (a) Schematic illustration of the experimental
protocol used to generate BM chimeric mice. Cd45.1+/Cd45.2+ recipient mice were lethally...
irradiated and then i.v. injected with CD45.1+ BM donor cells. Reconstituted BM chimeric mice were analysed after 10 weeks. (b) Representative plots showing the proportion of CD45.1+ cells and CD45.1+CD45.2+ cells within each myeloid cell subset analysed. The bar chart summarizes the percentages of CD45.1+ cells (black bar) and of CD45.1+/CD45.2+ cells (white bar) for each cell subset (n=1). Gating strategy is shown in Figure 3.5. The irradiated and reconstituted mice were provided by Dr. Sheng Jianpeng.

It is clear that in the LP of chimeric mice monocytes (P1) and monocyte-derived macrophages P2-P3 are fully derived from CD45.1+ precursors of the donor BM, since all cells are CD45.1+. As expected, almost 90% of F4/80 hi MHCII hi resident macrophages were BM-derived with only 10% of recipient origins, whereas F4/80 hi MHCII lo resident macrophages displayed a ratio 1:1 between donor and recipient cells, suggesting that about half of this cell population was refilled by the donor BM, while the remaining cells originated from the recipient and were still preserved after irradiation (Figure 3.23b).

3.2.6 The maintenance of tumour-resident macrophages does not require BM input in DSS-induced tumours

After successful characterization of the turnover rates of distinct myeloid subpopulations residing in the colon LP, we subsequently aimed to determine whether in the tumour environment the accumulation of F4/80 hi macrophages was due to an increased self-renewal or to an enhanced recruitment of newly generated BM-derived cells.

To trace the origins of myeloid cells in LP and tumour, Kit MerCreMer/R26 mice and Kit MerCreMer/R26 Apc Min/+ mice were firstly administered DSS in drinking water. Four weeks post-DSS treatment the YFP labelling was induced by injecting tamoxifen. Since it is well established that intestinal macrophages have a half-life of 3-5 weeks (107), we also have shown that the majority of F4/80 hi MHCII hi resident macrophages turned over after 4-7 weeks (Figure 3.22).

We decided to set a 7 week-time window of BM-precursor tracing in order to achieve a high YFP labelling for resident macrophages. After 7 weeks post- tamoxifen injection colon LP and tumours were analysed for the YFP index of each myeloid cell subset. Later timepoints of collection were not feasible because of animal ethical aspects.

The adult fate mapping analyses revealed that the recruitment rate of new cells into the tumours was not increased. The BM dependency of F4/80 hi MHCII lo macrophages was minimal and comparable to that one of the LP. A decrease in YFP labelling was observed in tumour-resident F4/80 hi MHCII hi macrophages when compared to their LP counterparts. This is probably due to the fact that the labelling mainly had occurred
before these cells were entrapped within the tumour niche and became independent from BM input (Figure 3.24a).

Figure 3.24: Different BM dependency of colon macrophage subsets in LP and DSS-induced tumour. (a) DSS-induced colon tumour formation was combined with adult fate mapping using Kit\(^{MerCreMer/R26}\) and Kit\(^{MerCreMer/R26}\)Apc\(^{Min+}\) mice (schematic representation shown in upper panel). After establishing the tumour, tamoxifen was injected for 5 consecutive days (1 dose/day) and the labelling percentage was assessed after 7 weeks. A total of 15 Kit\(^{MerCreMer/R26}\)Apc\(^{Min+}\) and 20 Kit\(^{MerCreMer/R26}\) were analysed. (b) For the embryonic fate mapping,
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pregnant Kit<sup>MerCreMer/R26</sup> and Kit<sup>MerCreMer/R26Apc<sup>Min+</sup></sup> mice at E10.5 were administered tamoxifen through one i.p. injection, as schematically illustrated in the upper panel. Eight-week-old offspring were then administered DSS and sacrificed 4 weeks later to determine the YFP labelling. A total of 4 Kit<sup>MerCreMer/R26Apc<sup>Min+</sup></sup> and 6 Kit<sup>MerCreMer/R26</sup> mice were analysed. For both (a) and (b): representative flow cytometry analysis shows the labelling efficiency for each myeloid cell subset. Gating strategy is shown in Figure 3.5. The bar chart represents the mean percentage of YFP<sup>+</sup> cells after normalization to the percentage of YFP<sup>+</sup> neutrophils. The error bars represent the s.e.m. E: embryonic day; P1: monocytes; P2 and P3: monocyte-derived macrophages. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. ***P <0.001. For reasons of clarity, the non-significant differences between groups were not indicated. The embryonic labelling via tamoxifen injection in pregnant mice was performed by Dr. Sheng Jianpeng.

Notably, when tamoxifen was administered during the embryogenesis at E10.5, all myeloid cell subpopulations, including tissue-resident macrophages in the healthy LP and colon tumours, exhibited similar efficiency of YFP labelling (Figure 3.24b), Indeed, all these myeloid cells had prenatal origins and were the progeny of Kit<sup>+</sup> embryo-derived precursors, as previously described (40).

3.2.7 The maintenance of tumour-resident macrophages does not require BM input in spontaneous tumours

The labelling efficiency of each myeloid cell subset was also assessed in spontaneously formed tumours. As shown in Figure 3.25, 4-month-old tumour-bearing mice had an average of 2 tumours of about 3 mm in diameter. Hence, 4-month-old Kit<sup>MerCreMer/R26</sup> and Kit<sup>MerCreMer/R26Apc<sup>Min+</sup></sup> were administered 5 consecutive doses of tamoxifen and analysed 4 months thereafter, since Apc<sup>Min+</sup> mice without DSS treatment do not develop DSS-induced colitis and high tumour burden, and are therefore able to live considerably longer (up to 12 months old) (Figure 3.26a).

![4-month-old Apc<sup>Min+</sup> mice](image)

Figure 3.25: Spontaneous colon tumours developed in almost all Apc<sup>Min+</sup> at 4 months old. From the left, bar showing the mean percentage ± s.e.m. of Apc<sup>Min+</sup> mice bearing tumours at 4 months of age (n=12). Dot plot graphs with the total number of polyps in each colon analysed and the tumour size of each polyp (measured as length of diameter in millimetres) respectively.

Four months post-tamoxifen administration, in LP all myeloid cell populations were completely YFP labelled, including F4/80<sup>+</sup>MHCII<sup>+</sup> resident macrophages. In aged mice, such as those of this experiment, F4/80<sup>+</sup>MHCII<sup>+</sup> macrophages became undetectable, hence were not included in the analysis of YFP labelling. In the tumour, we observed a
similar labelling pattern that we obtained in DSS-induced tumours. Both tumour-resident macrophage subsets did not reach a complete labelling (60% of YFP+ cells within MHCIIhi macrophages and 40% of YFP+ cells within MHCIIlo macrophages). This further indicated that in the tumour microenvironment the refilling of BM-derived F4/80hi resident macrophages was significantly less dependent on the BM input (Figure 3.26b).

Figure 3.26: Different BM dependency of colon macrophage subsets in LP and spontaneous tumour. (a) Schematic illustration of the experimental protocol (upper panel) and representative flow cytometry analysis with the labelling efficiency for each myeloid cell subset in LP (left) and in tumour (right). Gating strategy is shown in Figure 3.5. (b) The bar chart represents the mean percentage of YFP+ cells in each cell population after normalization to the percentage of YFP+ neutrophils. The error bars represent the s.e.m. Black bars: LP (n=4); white bars: tumour (n=3). Statistical significance was determined by two-way ANOVA followed by Bonferroni test. *P <0.05. For reasons of clarity, the non-significant differences between groups were not indicated. (Abbreviation: n.d.: not detectable).

3.2.8 Ccr2-independent resident macrophages do not require BM input

It is well documented and confirmed by our data (Figure 3.16 and 3.19), that in the intestine the CCR2-CCL2 axis plays a crucial role in the recruitment into the tissue of blood monocytes, which subsequently undergo a differentiation process into macrophages through short-lived intermediates (59). To further investigate this aspect in more detail, we performed a fate mapping experiment by utilizing KI/+MerCreMer/R26 mice
lacking CCR2 to demonstrate that in the LP, within the F4/80hi tissue-resident macrophages, CCR2-independent MHCIIlo and MHCIIIhi macrophages were minimally derived from BM precursors. Furthermore, in CCR2-deficient mice it was possible to analyse the YFP labelling for F4/80hi tumour-resident macrophages in absence of CCR2+ monocytes, which might be crucial for refilling F4/80hiMHCIIIhi macrophages in early tumoural stages, characterized by an immature microenvironment.

The applied experimental protocol was identical to that one in Figure 3.24a, with the exception that KitMerCreMer/R26 mice and KitMerCreMer/R26 ApcMin/+ mice were crossed with the Ccr2−/− mouse strain to obtain an adult fate mapping model in absence of CCR2.

**Figure 3.27:** Identification of BM-independent colon macrophage subsets in healthy and tumour tissue in absence of CCR2. (a) DSS-induced tumour formation was combined with the impairment of CCR2-CCL2 axis as well as the adult fate mapping, using the KitMerCreMer/R26 Ccr2−/− and KitMerCreMer/R26 Ccr2−/− ApcMin/+ mice (schematic representation shown in upper panel). For both (b) and (c): representative flow cytometry analysis illustrates the colon myeloid cell subset labelling efficiency. Gating strategy is shown in Figure 3.5. The bar chart represents the mean percentage of YFP+ cells after normalization to the percentage of YFP+ neutrophils ± s.e.m. In (b): LP percentages from Ccr2−/− mice (white bars) are compared with LP percentages of WT mice (black bars), obtained from Figure 3.24a. In (c): tumour percentages from Ccr2−/− ApcMin/+ mice (white bars) are compared with tumour percentages of ApcMin/+ mice (black bars), obtained from Figure 3.24a. A total of 10 KitMerCreMer/R26 Ccr2−/− ApcMin/+ and 8 KitMerCreMer/R26 Ccr2−/− mice were subjected to DSS treatment. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. *P<0.05; **P <0.01. For reasons of clarity, the non-significant differences between groups were not indicated. P1: monocytes; P2 and P3: monocyte-derived macrophages.
In **Figure 3.27b** the percentage of YFP+ cells for each cell subset in Ccr2−/− LP was compared to that one previously described in LP (Figure 3.24a). In the LP of Ccr2−/− mice, neutrophils and eosinophils were fully labelled, as they were not affected by the impairment of CCR2-CCL2 axis. The detectable monocytes and P2-P3 macrophages were completely YFP+, suggesting that they derived from BM progenitors and likely infiltrated the LP via other recruitment axes. As expected, the sparse CCR2-independent F4/80hiMHCIIlo macrophages were characterized by an even lower YFP index (about 10% of MHCIIlo cells were YFP+) whereas those tissue-resident F4/80hiMHCIIhi macrophages not affected by the absence of CCR2 displayed a significantly reduced YFP labelling (from 70% in LP to 20% in Ccr2−/− LP). This suggested that MHCIIlo LP-resident macrophages contained a minor CCR2-independent cell fraction that was negligibly replaced by BM-derived cells. In the tumour, intratumoural F4/80hiMHCIIhi macrophages were similarly YFP labelled when compared to F4/80hiMHCIIlo macrophages (20% of YFP+ cells within the subset, respectively), indicating that by preventing the early influx of CCR2-dependent F4/80hiMHCIIhi macrophages in the tumour, the resident macrophage pool in a mature tumour environment was mainly independent from BM input (**Figure 3.27c**).

### 3.2.9 Tissue-resident macrophage depletion enables fast refilling by BM-derived cells

Subsequently, we aimed to determine whether BM progenitors were capable to refill rapidly both F4/80hi macrophage subpopulations when macrophage niches were “artificially” emptied. In order to selectively deplete tumour-resident F4/80hi macrophages, we utilized a CD169-DTR mouse strain, previously generated in our laboratory (289). In CD169-DTR mice, upon DT injection tissue-resident macrophages are effectively ablated in various organs (288,289).

In **Figure 3.28a**, from the RNAseq DEGs it is clear that LP and tumour F4/80hi tissue-resident macrophages exclusively expressed Siglec1 (Cd169), whereas monocytes and neutrophils did not. Furthermore, when DT was injected twice before assessing the depletion efficiency, only F4/80hiMHCIIlo and F4/80hiMHCIIhi intratumoural macrophages were significantly depleted (about 70-80% of depletion) while the other myeloid cell subsets were not affected in their numbers (**Figure 3.28b**).

To quantify the BM dependency of those macrophages that refilled the tumour niche after DT-induced depletion, CD169-DTR KitMerCreMer/R26ApcMin/+ and CD169-DTR KitMerCreMer/R26 mice were firstly subjected to DSS treatment in order to establish the tumour microenvironment. Subsequently they were administered tamoxifen to induce the YFP labelling. Finally, 4 weeks after the first tamoxifen dose, were injected with 2
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doses of DT for 2 consecutive days (1 dose/day). The analysis was performed 4 weeks after DT injection to ensure that the macrophage niches were refilled. The experiment shown in Figure 3.28c confirmed that all macrophages, which replenished the empty niches, derived from adult BM haematopoiesis since both F4/80\(^{hi}\)MHCII\(^{hi}\) and F4/80\(^{hi}\)MHCII\(^{lo}\) fractions in the LP as well as in the tumour were fully YFP labelled.
(at day -2 and day -1) to CD169-DTR Apc\textsuperscript{Min/+} and Apc\textsuperscript{Min/+} mice before the analysis (day 0) to confirm the specific ablation of intratumoral F4/80\textsuperscript{hi}CD11b\textsuperscript{hi}CD169\textsuperscript{+} macrophages. Representative flow cytometry plots are shown. (c) DSS-induced tumour formation was combined with adult fate mapping and CD169\textsuperscript{+} macrophage ablation using CD169-DTR \textit{Kl}\textsubscript{MerCreMer/R26} and CD169-DTR \textit{Kl}\textsubscript{MerCreMer/R26} Apc\textsuperscript{Min/+} mice (schematic protocol in the upper part). Representative flow cytometry analysis illustrates the labelling efficiency for each cell subset. (d) The bar chart represents the mean percentage of YFP\textsuperscript{+} cells after normalization to the percentage of YFP\textsuperscript{+} neutrophils ± s.e.m. A total of 19 CD169-DTR \textit{Kl}\textsubscript{MerCreMer/R26} and 7 CD169-DTR \textit{Kl}\textsubscript{MerCreMer/R26} Apc\textsuperscript{Min/+} mice were analysed. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. For reasons of clarity, the non-significant differences between groups were not indicated. P1: monocytes; P2 and P3: monocyte-derived macrophages. Gating strategy is shown in Figure 3.5.

3.2.10 The maintenance of intratumoral resident macrophages requires CSF1R-CSF1 axis

After having demonstrated that CCR2-CCL2 axis was not required for the maintenance and accumulation of intratumoral F4/80\textsuperscript{hi} macrophages in colon adenomas, we decided to investigate the role of CSF1R-CSF1 axis. CSF1 is a crucial growth factor for macrophage proliferation and survival and it is shown to support the self-maintenance of tissue-resident macrophages (72,74,109). In Figure 3.29a, we compared the RNA levels of \textit{Csf1} in LP and colon tumours of various sizes and detected a progressive increase in the RNA expression of \textit{Csf1} as a function of the tumour progression. The RNaseq revealed that MHCII\textsuperscript{hi} and MHCII\textsuperscript{lo} resident macrophages sorted from DSS-accelerated tumours had higher expression of \textit{Csf1r} compared to their counterparts from the LP, as well as intratumoural and LP monocytes. Neutrophils from both tissues had the lowest RNA relative expression (Figure 3.29b).

To assess the influence of CSF1 in the maintenance of myeloid cell pools in both LP and tumour, we neutralized CSF1 by injecting an anti-CSF1R blocking antibody in DSS-treated WT and Apc\textsuperscript{Min/+} mice at day-4 and day-1 before harvesting the LP and colon tumours. In the LP, while eosinophils and neutrophils did not change in their numbers, F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages, as well as monocytes and P2-P3 macrophages were significantly reduced in numbers. Only F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} macrophages did not seem to require CSF1. In tumours, we observed a strong reduction in the absolute numbers of both F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} and F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} tumour-resident macrophages, whereas the numbers of neutrophils, monocytes and monocyte-derived macrophages were unperturbed and eosinophils even increased in their numbers (Figure 3.29d-e).
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Figure 3.29: F4/80<sup>hi</sup> resident macrophages depend on CSF1. (a) Intratumoural Csf1 RNA levels gradually increase with tumour progression. Colon LP and polyps of different sizes (ranging from 0.5 mm to 6 mm in diameter) were processed for quantitative PCR analysis of Csf1 expression. Data were normalized to β-actin and are expressed as fold-change in gene expression from the expression levels in 0.5 mm tumours. (b) Heat map showing the relative expression of Csf1r as log2 transformed RPKM in sorted cell subsets (MHCII<sup>hi</sup> and MHCII<sup>lo</sup> macrophages, monocytes, neutrophils) from LP and tumour. (c) 12-week-old DSS-treated Apc<sup>Min/+</sup> mice were i.p. injected with rat IgG2a or anti-CSF1R blocking antibody (400 µg/mouse) at day -4 and -1 before collection of the colons, as shown in the schematic representation. (d) Anti-CSF1R antibody partially depletes LP F4/80<sup>hi</sup>MHCII<sup>hi</sup> macrophages and P1, P2, P3 subsets. In the right panel, the bar charts show mean ± s.e.m. of absolute cell numbers. Control mice: n=6; anti-CSF1R Ab injected mice: n=5. (e) Two injections of anti-CSF1R are sufficient to drastically deplete intratumoural F4/80<sup>hi</sup> macrophages. Bar charts represent the mean ± s.e.m.
of absolute cell numbers. Control mice: n=6; anti-CSF1R Ab injected mice: n=5. For both (d) and (e): statistical significance was determined using an unpaired Student’s t-test. **P < 0.001; ***P < 0.0001; ns: not significant. Gating strategy is shown in Figure 3.5. (Abbreviation: Ab: antibody).

3.2.11 The maintenance of LP- and tumour-resident macrophages requires CSF1R-CSF1 axis

![Graph showing the maintenance of LP- and tumour-resident macrophages](Image)

**Fig. 3.30:** F4/80<sup>hi</sup> resident macrophages, and partially monocytes and monocyte-derived macrophages, depend on CSF1. (a) 10-week old DSS-treated Apc<sup>Min/+</sup> mice were i.p. injected with anti-rat IgG2a or anti-CSF1R blocking antibody (400 μg/mouse) for two consecutive weeks, alternate days, before collection of the colon, as shown in the schematic representation. (b) Comparison of the body weight curve of control Apc<sup>Min/+</sup> mice (light blue line) with that one of Apc<sup>Min/+</sup> mice injected with anti-CSF1R antibody (black line), from the DSS treatment to the day before the analysis. (c) Anti-CSF1R antibody fully depletes LP F4/80<sup>hi</sup> macrophages and partially depletes LP P1, P2, P3 subsets. In the right panel, the bar charts show the mean ± s.e.m. of absolute numbers (control mice: n=6; anti-CSF1R Ab injected mice: n=5). (d) Anti-CSF1R
antibody depletes intratumoural F4/80hi MHCIIhi and F4/80hi MHCIIhi macrophages, and partially P1, P2 and P3. Polyps were processed and cells were analysed by flow cytometry to detect different myeloid cell populations defined in Figure 3.5. In the right panel, the bar charts show the mean ± s.e.m. of absolute numbers (control mice: n=6; anti-CSF1R Ab injected mice: n=5). For (c) and (d) statistical significance was determined using an unpaired Student’s t-test. *P < 0.05, **P < 0.001, ***P < 0.0001, ns: not significant. (Abbreviation: Ab: antibody).

To evaluate the effects of a prolonged CSF1R-CSF1 axis impairment in the myeloid cell compartment from LP and tumour, we injected anti-CSF1R antibody for 2 weeks after DSS treatment, on alternate days, until the day before harvesting the colons. The body weight of WT and ApcMin/+ mice was monitored throughout the experimental protocol, from the beginning of DSS treatment to the last day of anti-CSF1R antibody injection (day 34) to demonstrate that CSF1R blocking treatment did not induce any body weight reduction and was perfectly tolerated, when compared to the control group of mice injected with anti-IgG2a. In the LP of mice injected with anti-CSF1R antibody a total ablation of F4/80hi MHCIIhi was this time observed; moreover F4/80hi MHCIIhi were also significantly reduced in their numbers, as well as monocytes and P2-P3 macrophages. In contrast, eosinophils markedly increased in numbers (Figure 3.30c). In the tumour, the prolonged treatment completely depleted both F4/80hi macrophage subsets, and P1-P3 cells were partially affected as well (Figure 3.30d).

3.2.12 Intratumoural MHCIIlo and MHCIIhi macrophages are functionally similar

In order to determine whether the accumulation of tissue-resident macrophages within the tumour environment was likely due to a phenotypic change with gain-of-function in the capability to proliferate, we analysed the transcriptome of both intratumoural F4/80hi macrophage populations, neutrophils and monocytes, and the corresponding subsets from the LP. Dendrogram (Figure 3.31a, right panel) and three-dimensional principal component analysis (3D-PCA) (Figure 3.31a, left panel) of all expressed genes revealed that each myeloid cell fraction from the LP showed a distinct and separated transcriptomic profile, with F4/80hi MHCIIhi and F4/80hi MHCIIhi macrophage subsets clustering very closely. Furthermore, all intratumoural cell subsets were clearly clustered separately from their LP counterparts, indicating that the tumour microenvironment could be the driving force that shaped the transcriptome of tumour-infiltrating myeloid cells. Similarly to the LP, both intratumoural F4/80hi MHCIIhi and F4/80hi MHCIIhi macrophage subsets were closely grouped, suggesting a similar transcriptional profile. Venn diagram (Figure 3.31b) and Volcano plots (Figure 3.31c) highlighted only 212 genes as differentially expressed (133 upregulated and 79 downregulated) between intratumoural MHCIIlo and MHCIIhi macrophages, whereas the LP counterparts shared 1,902 differentially expressed genes (DEGs), 1,073
upregulated and 829 downregulated. When the comparison was performed between LP and tumour MHCII\textsuperscript{lo} subsets, as well as between LP and tumour MHCII\textsuperscript{hi} subsets, DEGs were considerably more (3,848 and 4,324 respectively). Within the 212 DEGs between the two tumour-resident macrophage subsets, Arg1 and Marco were upregulated in MHCII\textsuperscript{lo} macrophages, whilst genes related to the MHC such as Ciita, H2-Ab1, H2-Eb1 and H2-Aa were downregulated. Indeed, this latter differential expression enabled to clearly separate F4/80\textsuperscript{hi} intratumoural macrophages based on extracellular MHCII expression by flow cytometry.

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**Figure 3.31: The transcriptome of intratumoural F4/80\textsuperscript{hi} macrophage subsets is similar.**

(a) 3D-PCA (left) including the 4 cell subsets (monocytes, neutrophils, F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} and F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} macrophages) obtained from colon LP and their counterparts from the tumour. The dendrogram (right) illustrates the hierarchical clustering of the same sorted myeloid cell populations. Labelling colours used: grey: tumour neutrophils; wine red: LP neutrophils; light blue: tumour monocytes; light green: LP monocytes; beige: tumour MHCII\textsuperscript{lo} macrophages; pink: tumour MHCII\textsuperscript{lo} macrophages; red: LP MHCII\textsuperscript{lo} macrophages; blue: LP MHCII\textsuperscript{lo} macrophages.

(b) Venn diagram shows the number of DEGs between F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} and F4/80\textsuperscript{hi}MHCII\textsuperscript{lo}
subpopulations in LP and tumour, respectively. Venn diagram was generated with the upregulated and downregulated DEGs from the following two comparisons: tumour MHCII\(^{hi}\) versus MHCII\(^{lo}\) macrophages and LP MHCII\(^{lo}\) versus LP MHCII\(^{hi}\) macrophages. (c) Volcano plots compare DEGs between the 4 possible combinations of F4/80\(^{hi}\) resident macrophage subsets from LP and tumour. Dot plots were generated with log\(_{2}\)FC in X-axis and –log\(_{2}\)(pValue) in Y-axis. The volcano plot comparing MHCII\(^{lo}\) and MHCII\(^{hi}\) intratumoural macrophage subpopulations is visualized in blue. Two upregulated genes in MHCII\(^{lo}\) macrophages, such as Marco and Arg1 (in green), and 4 upregulated genes in MHCII\(^{hi}\) macrophages, such as Cilia, H2-Ab1, H2-Eb1 and H2-Aa (in red), are highlighted. Myeloid cell subpopulations were sorted from pooled colons and pooled tumours obtained from 13-15 mice, respectively, in 3 independent experiments. The gating strategy shown in Figure 3.17b enabled to sort the following subsets with high purity: F4/80\(^{+}\)CD11b\(^{+}\)Ly6C\(^{+}\)MHCII\(^{hi}\) monocytes, F4/80\(^{+}\)CD11b\(^{-}\)neutrophils, F4/80\(^{+}\)CD11b\(^{-}\)MHCII\(^{hi}\) and F4/80\(^{+}\)CD11b\(^{-}\)MHCII\(^{lo}\) tissue-resident macrophages. (Abbreviations: FC: Fold Change; log: logarithm; T: tumour; LP: Lamina Propria).

3.2.13 Intratumoural MHCII\(^{lo}\) and MHCII\(^{hi}\) macrophages self-maintain through in situ proliferation

In Figure 3.32 we have demonstrated that in the tumour environment F4/80\(^{hi}\) macrophages expanded their cell pools via in situ proliferation, which was not the case for their counterparts in the LP. Tumour-resident macrophages were clearly in an active phase of the cell cycle (G\(_{1}\), S, G\(_{2}\), and mitosis), as indicated by the expression of Ki67, which is a nuclear protein expressed during all active phases of the cell cycle, but is absent in quiescent cells (G\(_{0}\)). About 20% of MHCII\(^{lo}\) and MHCII\(^{hi}\) intratumoural macrophages were indeed Ki67\(^{+}\) (Figure 3.32a). Moreover, the RNAseq heat map and the real time qPCR indicated a clear upregulation of several cell cycle regulators, including cyclin-dependent kinase 1 (Cdk1), Cell Division Cycle 8a, 20 and 25C proteins (Cdc8a, Cdc20 and Cdc25c), cyclins such as cyclin-A2 and –B2 (Ccn2, Ccnb2), E2F transcription factor 2 (E2f2), NEK family of serine/threonine kinases such as Nek2 and Nek3, various Minichromosome maintenance complex components (Mcm2, Mcm3, Mcm5, Mcm7) as well as marker of proliferation Ki67 (Mki67) (Figure 3.32b). Interestingly, in the LP F4/80\(^{hi}\)MHCII\(^{hi}\) and F4/80\(^{lo}\)MHCII\(^{lo}\) macrophages were not in an active phase of the cell cycle (Figure 3.32a-b). The lack of self-renewing capability might explain the gradual disappearance of MHCII\(^{lo}\) macrophages in the LP, at the onset of the adulthood in mice, since they were extremely slowly replaced by BM-derived cells (Figure 3.13 and Figure 3.22). On the contrary, since the LP F4/80\(^{hi}\)MHCII\(^{hi}\) macrophage pool was mainly maintained by a constant recruitment of BM-derived precursors, the refilling with BM-derived macrophages seemed to prevail over the situ self-renewal.
Figure 3.32: In the colon tumour, tissue-resident macrophages expand their cell numbers via in situ proliferation. (a) The proliferative capability of F4/80<sup>hi</sup> tissue-resident macrophage subpopulations in LP and tumour was determined by flow cytometry staining of intracellular expression levels of Ki67. Representative plots showing the expression of Ki67 within each cell subset tested. The complete gating strategy is reported in Figure 3.5. The bar chart (right panel) summarizes the percentage of Ki67<sup>+</sup> cells within each cell subset, in colon LP obtained from DSS-treated WT mice (n=5, black bars) and DSS-induced colon tumours of Apc<sup>Min/+</sup> mice (n=10, white bars). Error bars represent the s.e.m. Statistical significance was determined by two-way ANOVA followed by Bonferroni test; **P < 0.01; ***P < 0.001; ns: not significant. (b) Heat map of cell cycle-associated genes in intratumoural F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> subsets, and the corresponding subsets from the LP. The heat map was generated with log2 transformed RPKM values and with the row/gene median subtracted. The targets marked in red were validated by qPCR. (Red: upregulated; blue: downregulated). (c) The 4 macrophage subsets were sorted to obtain the RNA for qPCR analysis to validate the relative expression of several target genes of the RNAseq heat map. Data were normalized to the expression levels of β-actin and were expressed as fold-change from the corresponding expression level in LP MHCII<sup>hi</sup> macrophages. (Black bars: LP; white bars: tumour. Abbreviation: n.d.: not detectable).
3.3 Functional characterization of tumour-resident macrophages

3.3.1 Pro-tumoural properties are restricted to F4/80<sup>hi</sup> macrophages

In order to have a macroscopic evaluation of a pro-tumoural or anti-tumoural potential of different types of tumour-infiltrating macrophages, we induced the tumorigenesis in Apc<sup>Min/+</sup> mice lacking distinct subpopulations of myeloid cells. Apc<sup>Min/+</sup> mice subsequently treated with anti-CSF1R antibody, Ccr2<sup>+/-</sup> Apc<sup>Min/+</sup> mice and, as a control, Csf2r<sup>-/-</sup> Apc<sup>Min/+</sup> mice were analysed (Figure 3.33 and Figure 3.34).

![Diagram](image)

**Figure 3.33:** CSF2R deficiency and CCR2 deficiency do not reduce the tumour burden in Apc<sup>Min/+</sup> mice, respectively. (a) Comparison of the body weight curve of Apc<sup>Min/+</sup> mice (black line) with that one of Csf2r<sup>-/-</sup> Apc<sup>Min/+</sup> mice (light grey line) during the experiment, from the DSS
treatment to the day before the analysis. (b) Graphs showing the mean ± s.e.m. of the total number of polyps per colon, the number of polyps with a diameter less than 2 mm and more than 2 mm in Apc<sup>Min/+</sup> (black dots, n=16) and Csf2<sup>R−/−</sup>Apc<sup>Min/+</sup> (white dots, n=13) mice. (c) Comparison of the body weight curve of Apc<sup>Min/+</sup> mice (black line) with that of one of Ccr2<sup>−/−</sup>Apc<sup>Min/+</sup> mice (light grey line). (d) Identical analysis described in (b) with 9 Apc<sup>Min/+</sup> mice (black dots) and 9 Ccr2<sup>−/−</sup>Apc<sup>Min/+</sup> mice (white dots). Statistical significance was determined using an unpaired Student’s t-test. Non-significant difference was not indicated. The experiment in Figure 3.33b was performed in collaboration with Chen Qi.

At the same timepoint (4 weeks after DSS treatment) colons were collected to perform the polyp count and the measurement of tumour size. Only those mice that had a comparable DSS-induced body weight reduction at day 9-10, which is likely proportional to the severity of DSS-induced epithelial damage and acute inflammation, were included in the analysis (body weight curves in Figure 3.33 and Figure 3.30b). CSF2R deficiency was previously shown to affect exclusively the infiltration of eosinophils, both in the LP and tumour. On the contrary, F4/80<sup>hi</sup> tissue-resident macrophages and F4/80<sup>int</sup> monocyte-derived macrophages (P2-P3) did not change in their absolute numbers. In the analysis of tumour load and progression, Csf2<sup>R−/−</sup>Apc<sup>Min/+</sup> mice carried a comparable number of polyps throughout the colon, with similar size, when compared to Apc<sup>Min/+</sup> littermates (Figure 3.33a). Clearly, CCR2 deficiency, although strongly associated with reduced monocytes and monocyte-derived macrophages, did not cause any change on polyp numbers in Ccr2<sup>−/−</sup>Apc<sup>Min/+</sup> mice compared to their Apc<sup>Min/+</sup> littermates (Figure 3.33b). Taken together, CSF2R-CSF2 axis and CCR2-CCL2 axis did not seem to be involved in recruiting and/or sustaining tumour-promoting myeloid cells within the tumour environment.

**Figure 3.34: CSF1R blockade significantly reduces the tumour burden in Apc<sup>Min/+</sup> mice.** (a) Schematic representation of the experimental protocol used. Two weeks after DSS treatment Apc<sup>Min/+</sup> mice that had a similar DSS-induced body weight reduction and recovery phase were divided into 2 groups of treatment and either injected with anti-rat IgG2a isotype antibody (control group) or with anti-CSF1R antibody. The body weight curve of the experiment is
Results

reported in Figure 3.30b. Representative pictures with 2 colons from the control group and 2 colons from the anti-CSF1R injected group. (b) From the left, total number of polyps in the colon of anti-rat IgG2a injected Apc\textsuperscript{Min/+} (black dots, n=11) and anti-CSF1R Ab injected Apc\textsuperscript{Min/+} mice (white dots, n=9). The polyps were separated into smaller than 2 mm and bigger than 2 mm in diameter, and counted according to these criteria. Statistical significance was determined using an unpaired Student's t-test. *P < 0.05 and **P < 0.001. (Abbreviation: Ab: antibody).

In contrast, the 2-week depletion of tumour-resident F4/80\textsuperscript{hi} macrophages using anti-CSF1R blocking antibody triggered a significant reduction of the total number of polyps in the colon, furthermore the detected tumours were smaller in diameter (Figure 3.34b). These observations suggested that particularly F4/80\textsuperscript{hi} macrophages could be involved in tumour-promoting functions in an established tumour microenvironment.

3.3.2 Metabolic switch of intratumoural F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} and F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages

In colon tumours, besides the acquisition of self-renewal potential, F4/80\textsuperscript{hi} tissue-resident macrophages gained a novel metabolic signature that was markedly distinct when compared to the cell counterparts in healthy colon LP. RNAseq analyses revealed that various RNA transcripts of the glycolytic pathway were upregulated, as well as transcripts of the urea cycle and those involved in glycogen and triglyceride metabolism, and nucleotide synthesis.

In order to fulfil their bioenergetics demands to rapidly proliferate, malignant epithelial cells undergo a metabolic reprogramming, in which the aerobic glycolysis with lactate synthesis is favoured (Warburg effect) (320). Similarly, tissue-resident macrophages in the tumour environment increased the transcript levels of glycolytic genes such as Enolase 1 (Enol1), Triosephosphate Isomerase 1 (Tpi1), Phosphoglycerate Mutase 1 (Pgami), Pyruvate Kinase Muscle (Pkm), Phosphoglycerate Kinase 1 (Pgk1), Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh), Lactate Dehydrogenase A (Ldha) and Pyruvate Dehydrogenase Alpha 1 (Pdha1), with F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} macrophages characterized by the highest levels. The expression of urea cycle genes, such as Arginase 2 (Arg2) and Arginosuccinate synthetase 1 (Ass1) was upregulated in tumour-resident macrophages as well (Figure 3.35a-b). When we considered a typical list of markers for alternatively activated macrophages (so-called M2-like) that might be expressed in our F4/80\textsuperscript{hi} TAMs, we observed that both tumour-resident macrophage subsets upregulated Arginase 1 (Arg1) and Macrophage receptor with collagenous structure (Marco), with MHCII\textsuperscript{lo} macrophages being the major producers (Figure 3.35c-d). The upregulation of Arg1 was also confirmed at the protein level by flow cytometry (Figure 3.35e).
Results

Figure 3.35: Intratumoural F4/80$^\text{hi}$ macrophages upregulate the expression of glycolytic genes, *Marco* and *Arg1*. (a) Heat map of the relative expression of glycolysis- and urea cycle-associated genes, in (from the left) LP MHCII$^\text{lo}$ and MHCII$^\text{hi}$ macrophages, intratumoural MHCII$^\text{hi}$ and MHCII$^\text{lo}$ macrophages. The heat map was generated with log2 transformed RPKM values and with the row/gene median subtracted. The results of those targets marked in red were validated by qPCR. (b) The 4 macrophage subsets were sorted from pooled LP and tumours of 13-15 mice, respectively. Total RNA was extracted for real-time qPCR to validate the relative expression of several target genes from the RNAseq heat map. Data were normalized to β-actin and were expressed as fold-change from the corresponding expression level in LP MHCII$^\text{hi}$ macrophages. (Black bars: LP; white bars: tumour). (c) Heat map of the relative expression of M2-like macrophage targets. The heat map was generated with log2 transformed RPKM values and with the row/gene median subtracted. (d) Real-time qPCR of *Marco* and *Arg1* expression
levels in the LP and in intratumoural F4/80^hi/MHCII^hi and F4/80^hi/MHCII^lo subpopulations. Error bars represent the s.e.m. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. ***P<0.001; ns: not significant. (e) Representative flow cytometry histograms (left panel) and bar chart (right panel) showing the intracellular mean of fluorescence intensity (MFI) of the ARG1 expression in each macrophage subset, from LP and DSS-induced tumours. The grey histogram in each flow cytometry plot corresponds to the isotype control. Error bars represent the s.e.m. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. **P<0.01; ***P < 0.001; ns: not significant.

Of note, other classical markers for M2-polarized macrophages such as *Mrc1*, *Cd163* and Transglutaminase 2 (*Tgm2*) were not found enriched in the intratumoural macrophage subsets. This firstly suggests that the tumour environment could shape TAMs with a unique phenotype in vivo that may not strictly overlap to the phenotype of in vitro generated M2-like macrophages; secondly, TAM phenotype may differ depending on the tumour type, and within a certain tumour distinct TAM subsets may be phenotypically diverse, depending on the tumoural stage.

### 3.3.3 Tumour-induced metabolic reprogramming and phenotype switch particularly occurs in F4/80^hi/MHCII^lo macrophages

Although hierarchical clustering between distinct colon myeloid cells clearly identified F4/80^hi/MHCII^hi and F4/80^hi/MHCII^lo as demarcated populations, “high/low” pairs always formed more closely related couples in tumours or in the LP than “low/low” or “high/high” comparisons between subsets from different tissue. Although these data suggested a close relationship between F4/80^hi/MHCII^hi and F4/80^hi/MHCII^lo cells (Figure 3.31), ingenuity pathway analysis (IPA®) revealed that multiple pathways involved in glycolysis, gluconeogenesis, urea cycle, HIF-1α and colorectal cancer metastasis signalling were significantly upregulated in F4/80^hi/MHCII^lo cells (P < 0.05 by right-tailed Fisher’s exact test, Figure 3.36, red arrows). Clearly, tumour-resident F4/80^hi/MHCII^lo cells not only accumulated, but also were subjected to a robust metabolic reprogramming during tumour progression.
Figure 3.36: In the tumour, MHCII<sup>lo</sup> macrophages are characterized by a more robust metabolic reprogramming. IPA® of differentially expressed genes between intratumoural F4/80<sup>hi</sup>MHCII<sup>lo</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> macrophages. Bars indicate the P values (−log10) for
pathway enrichment (axis on the top). The pathways, shown as bars, are all significantly enriched with p-value < 0.05 (-log10 p-value > 1.301). Red arrows highlight pathways involved in glycolysis, gluconeogenesis, urea cycle and colorectal cancer metastasis signalling.

### 3.3.4 Intratumoural F4/80<sup>hi</sup> macrophages as crucial players in ECM remodelling

**Figure 3.37:** Intratumoural F4/80<sup>hi</sup> macrophages acquire a distinct phenotype that potentially favours the tumour ECM remodelling. (a) The heat map illustrates the differential expression of genes involved in ECM remodelling, and was generated with log2 transformed RPKM values and row/gene median subtracted. In the transcriptome analysis 6 cell populations were included (from the left: tumour monocytes; LP monocytes; LP MHCI<sup>lo</sup>; LP MHCI<sup>hi</sup>; tumour MHCI<sup>lo</sup> and tumour MHCI<sup>hi</sup> macrophages). Targets highlighted in red were considered for the comparison between intratumoural monocytes and the two F4/80<sup>hi</sup> macrophage subsets in (c). (b) Validation of the relative expression patterns for Mmp2, Mmp9 and Mmp12 in LP and
intratumoural F4/80^{hi}MHCII^{hi} and F4/80^{hi}MHCII^{lo} subsets quantified by real-time qPCR. Error bars represent the s.e.m. Statistical significance was determined by two-way ANOVA followed by Bonferroni test. *P<0.05; ***P < 0.001; ns: not significant. (c) Intratumoural monocytes, MHCII^{hi} and MHCII^{lo} macrophages were compared for the relative expression levels of ECM remodelling-associated genes, highlighted in red in (a), through real-time qPCR.

Several transcripts of MMPs involved in the degradation of ECM, such as Mmp2, Mmp9 and Mmp12, were upregulated in both tumour-resident macrophage subpopulations when compared to their LP counterparts (Figure 3.37a-b), suggesting a possible contribution of F4/80^{hi} macrophages in the tumour progression by altering the matrix architecture to favour the metastatic spread of cancerous cells (151).

3.4 Macrophage-based immunotherapy in combination with chemotherapy for the treatment of colon cancer

3.4.1 Toxicity and side effects of high dose chemotherapy regimen in colon cancer model

Starting from the previously described observation that the prolonged treatment with anti-CSF1R antibody may induce the arrest and/or regression of the tumour growth in our experimental tumour model obtained in DSS-treated Apc^{Min/+} mice, we aimed to investigate whether combining this treatment with a chemotherapy regimen might lead to a more effective therapeutic outcome. The first experiment, reported in Figure 3.38, was performed to assess the tolerability and effectiveness of a high dose regimen with a combination of 3 chemotherapeutic drugs (100 mg/kg 5-Fluorouracil, 10 mg/kg Oxaliplatin, 100 mg/kg Irinotecan), i.p. injected once a week with the first dose 2 weeks after the DSS treatment. After the first injection, chemotherapy-treated Apc^{Min/+} mice were shown to have side effects associated to the toxicity of the treatment, such as evident body weight reduction, diarrhoea and discomfort. The second injected dose degenerated their health condition, leading to a 50% of survival rate and the necessity to discontinue the chemotherapy. The treatment was terminated at the second dose and the colons were collected at day 35 (Figure 3.38a-b). Despite being particularly toxic, a 2-week cycle of chemotherapy was extremely effective as the analysed colons were almost devoid of polyps (Figure 3.38c).
Figure 3.38: Toxicity of the chemotherapy in high dose regimen. (a) Comparison of the body weight curve of untreated Apc\textsuperscript{Min/+} mice (black line with black dots; n=5) with that of chemotherapy-treated Apc\textsuperscript{Min/+} mice (black line with white dots; n=4) during the experiment, from the DSS treatment to the day before the analysis. (b) Survival curve of untreated Apc\textsuperscript{Min/+} mice (solid line) and chemotherapy-treated Apc\textsuperscript{Min/+} (dashed line). (c) Graphs showing the mean ± s.e.m. of the total number of polyps per colon, the number of polyps with a diameter less than 2 mm and more than 2 mm in untreated Apc\textsuperscript{Min/+} (black dots; n=5) and Apc\textsuperscript{Min/+} mice survived to the chemotherapy (white dots; n=2). Statistical significance was determined using an unpaired Student’s t-test. Non-significant difference was not indicated. (Abbreviation: chemo: chemotherapy).

3.4.2 Anti-CSF1R treatment in combination with suboptimal chemotherapy did not robustly ameliorate the disease outcome

In the experiment reported in Figure 3.39 we intended to design a therapeutic approach to be used in DSS-treated tumour-bearing Apc\textsuperscript{Min/+} mice, by combining immunotherapy and chemotherapy. Specifically, the previously tested anti-CSF1R antibody injection scheme was associated with a suboptimal 3-week cycle of chemotherapy. To define a suboptimal dose of chemotherapy, we assessed the tolerability and efficacy of various 3-week regimens with more diluted drugs, injected once a week (data not shown). Similarly to the previous experiments of polyps count and comparison of treatments,
only those $Apc^{Min/+}$ mice with a comparable DSS-induced body weight reduction at day 9-10, which is likely proportional to the DSS-induced acute inflammation, were either administered the treatment or used as control untreated group. The dilution 1:5 of the high dose regimen (20 mg/kg 5-Fluorouracil, 2 mg/kg Oxaliplatin, 20 mg/kg Irinotecan) was tolerated and was not shown to successfully induce the tumour regression. Hence, after DSS-treatment at day 21, $Apc^{Min/+}$ were divided into 4 experimental groups, i.e. untreated control group, anti-CSF1R antibody injected group, chemotherapy-treated group with the suboptimal dose (20 mg/kg 5-Fluorouracil, 2 mg/kg Oxaliplatin, 20 mg/kg Irinotecan) and group treated with the combination of anti-CSF1R antibody and suboptimal chemotherapy. In the 2 groups treated with anti-CSF1R, the first dose was injected at day 21 to ensure that intratumoural F4/80$^{hi}$ macrophages were depleted by the beginning of the chemotherapy. Anti-CSF1R antibody was injected on alternate days till the day before the analysis. The chemotherapy was administered for 3 consecutive weeks, one dose weekly. The body weight was monitored on alternate days to evaluate potential signs of physical discomfort.

Figure 3.39: The combination of suboptimal chemotherapy and anti-CSF1R antibody has a weak effect on tumour regression. (a) Comparison of the body weight curve of untreated $Apc^{Min/+}$ mice (black line with black dots; n=22), anti-CSF1R injected $Apc^{Min/+}$ mice (light green line with light green dots; n=16), chemotherapy-treated $Apc^{Min/+}$ mice (black line with white dots; n=14) and $Apc^{Min/+}$ mice treated with the combination of chemotherapy and antibody (blue line with light blue dots; n=16) during the experiment, from DSS treatment to the day before the analysis (day 43). (b) Representative pictures of tumour-bearing colons, one for each condition of treatment. (c) Graphs showing the mean ± s.e.m. of the total number of polyps per colon, the number of polyps with a diameter less than 2 mm and more than 2 mm in untreated $Apc^{Min/+}$ mice (black dots; n=22), $Apc^{Min/+}$ mice treated with anti-CSF1R antibody (light green dots; n=16),
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\( Apc^{Min/+} \) mice under suboptimal chemotherapy (white dots; \( n=14 \)) and treated with the combination (light blue dots; \( n=16 \)). Statistical significance was determined using one-way ANOVA test followed by Bonferroni multiple comparison. *\( P<0.05 \); **\( P<0.01 \); ***\( P<0.001 \). Non-significant differences were not indicated. (Abbreviations: chemo: chemotherapy; Ab: antibody).

Despite being tolerated better than the high dose regimen, the suboptimal chemotherapy induced a detectable body weight reduction, in particular when combined with anti-CSF1R antibody treatment. On the contrary, anti-CSF1R treatment as monotherapy was again well tolerated (Figure 3.39a). In our experimental colon cancer model, we could not detect a robust reduction in the polyp count in the group of \( Apc^{Min/+} \) mice treated with the combined therapy when compared to the group of \( Apc^{Min/+} \) mice treated with the anti-CSF1R antibody alone. This suggests that in the presence of a suboptimal regimen of chemotherapy, the combination did not induce any synergic effect of tumour regression in these experimental settings. Nevertheless, a significant reduction in the number of small polyps (with a diameter less than 2 mm) was detected (Figure 3.39c).
4 DISCUSSION

4.1 Tumour-associated macrophages in colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide (321). CRC prevalence is higher in industrialized countries of North America, Europe and Australia. However, it is increasing in countries that have recently made a transition towards a high-income economy and have adopted western lifestyles, particularly in South America, Asia and Eastern Europe (322).

Non-modifiable and modifiable/environmental factors may contribute to CRC pathogenesis. Individuals with a personal history of benign adenomatous polyps or inflammatory bowel diseases (ulcerative colitis and Crohn’s disease) have a higher risk of CRC (323). Inherited genetic mutations can predispose to familial adenomatous polyposis (FAP) and Lynch syndrome (324). The major environmental risk factors are diet, physical activity, cigarette smoking and heavy alcohol consumption (325,326).

CRC has been referred as a genetic disease closely associated with chronic inflammation. Indeed, inflammatory bowel diseases may evolve to CRC (327,328). Tumour-promoting inflammation has become a new hallmark of cancer (134) and immune cells, including TAMs, which can infiltrate the tumour at all stages, appear to affect cancer growth and metastasis (329). Clinical studies on TAM abundance and patient prognosis have provided conflicting evidence for CRC (142-154), even though recent preclinical studies have shed light on TAM involvement in tumour-promoting mechanisms of ECM remodelling, angiogenesis and tumour growth (149-154) (Table 6).

<table>
<thead>
<tr>
<th>TAM function</th>
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<td>Harmful</td>
<td>Patient</td>
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<td>Harmful</td>
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<td>Harmful</td>
<td>Mice/Cell culture</td>
<td>Cancer cell invasion</td>
<td>152</td>
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<tr>
<td>Harmful</td>
<td>Cell culture</td>
<td>Cancer cell proliferation/invasion</td>
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**Table 6: Overall function of TAMs in CRC.** Some clinical studies have reported a beneficial role of TAMs. In contrast, in studies using either patient specimens or mouse models/in vitro systems the harmful function of TAMs promotes the tumour progression. Taken and adapted from Zhonga X., Chen B., Yanga Z. Cell Physiol. Biochem. 45, 356-365 (2018).

With regards to the clinical practice guidelines for CRC treatment, a large proportion of early stage patients are successfully cured with an aggressive surgical resection of the
primary tumour mass. Patients with stage IV disease, metastatic CRC and with high risk of relapse are subjected to chemotherapy as adjuvant treatment. In case of relapse, in a second-line treatment a targeted therapy is eventually added to chemotherapy in order to interrupt specific tumour-promoting mechanisms (330). Immunotherapy, which directly targets the immune system of the tumour microenvironment, is emerging as a novel therapeutic approach to achieve a durable anti-tumour response. Several therapeutic strategies targeting macrophages for their tumour-promoting contribution are undergoing clinical assessment (data available in http://www.clinicaltrials.gov).

In order to design successful TAM-targeting therapies, TAM ontogeny and mechanisms of refilling in the tumour are under investigation. To date, the lineage and maintenance of TAMs in colon cancer have not been studied. In order to address these questions, we have opted for an in vivo model of colon cancer (ApcMin/+ mouse model) which faithfully recapitulates the multistep process of tumorigenesis, including the chronic inflammation.

Human CRC is the result of a gradual accumulation of oncogenic gene mutations. A hyperproliferative epithelium evolves to benign adenoma, to eventually malignant carcinoma over a period of 10-40 years. The most common gene mutations occur in oncogenes (K-ras) and tumour suppressor genes (for example Apc and Tp53). Certain mutations can alter the protein expression of Cox2, β-catenin, iNOS and mediators of the Wnt/Apc/β-catenin signalling pathway (331). Furthermore, epigenetic modifications, such as changes in the DNA methylation status, can contribute to the intestinal tumorigenesis (332). In order to develop a malignant tumour at least 4-5 mutations are required, often in a preferential sequence. Fap and Apc genes are generally the first genes to be mutated in the transition from a normal epithelium to hyperproliferative epithelium, while p53 loss occurs in late adenomas (333) (Figure 4.1).

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**Figure 4.1: CRC as a multistep process of tumour progression.** Early alterations in genes of 5q chromosome, including Apc and Fap, induce the normal colonic epithelium to be hyperproliferative. DNA methylation and mutations in 12p (K-ras) promote the transition from early adenoma to intermediate adenoma, whereas mutations in 18q favour the progression to late adenoma. Loss of p53 is a late event leading to carcinoma. Other alternations confer to the carcinoma the potential to metastasize. (Abbreviations: p region: shorter arm of the chromosome; q region: longer arm of the chromosome; DCC: Deleted in Colorectal Cancer). Taken and adapted from Fearon E.R. and Vogelstein B. Cell. 61, 759-767 (1990).
Colon tumours spontaneously develop in Apc\textsuperscript{Min/+} mice when a stochastic mutation occurs in the WT allele of Apc. The accumulation of mutant APC triggers the onset of an uncontrolled proliferation in the colonic crypt (301,302). In the colon, we were able to observe occasional tumours that were generated from a multistep progression: from single aberrant crypt to adenoma, and rarely to carcinoma (Figure 3.1 and Figure 3.2). Despite of the limited number of macroscopic tumours, the loss of functional APC leads to the development of several microadenomas in the colon (334). The advantage of this transgenic mouse model is the gradual tumour formation, which is associated with a concomitant development of the tumour microenvironment. Furthermore, the tumours are intrinsically heterogeneous, since not derived from an inoculated homogenous cancer cell line (Figure 3.4). One disadvantage is the short lifespan of the mice (4-12 months), hence the adenomas generally do not acquire sufficient mutations to progress to carcinoma and metastasise (335,336). Furthermore, the tumours predominantly develop in the small intestine rather than in the colon. In order to overcome these two limitations, for all experiments we have exploited a colitis-induced CRC model, unless otherwise stated. DSS treatment in drinking water accelerates the onset of macroscopic tumours by inducing the breakdown of the mucosal epithelial barrier that results in an overwhelming inflammatory response. In our animal facility, we have established a protocol with DSS concentration at 1.5 % (w/v), so that after the 7-day DSS cycle generally all Apc\textsuperscript{Min/+} mice are able to recover and after one month carry several polyps of different sizes throughout the colon (Figure 3.3).

4.2 Skewed representation of myeloid cells in colon adenomas

It is well-documented that tumorigenesis is supported by the tumour microenvironment, which is a chaotic, dynamic and deregulated network of numerous types of stromal cells, including endothelial cells, fibroblasts and immune cells of both adaptive and acquired immunity as well as the ECM and the local milieu of soluble factors (131). Tumours in mice and humans are characterized by the infiltration of mature populations of TAMs, frequently comprising more than 50% of the leukocytes. Depending on the tumour area (i.e. hypoxic area, perivascular area, tumour-stroma border), TAMs have been described to play different functions, suggesting that a certain tumour may be populated by functionally distinct macrophage subsets (337). Limited studies have profiled the immune system in colon cancer. Akeus et al. have dissected the tumour-associated lymphocytes in Apc\textsuperscript{Min/+} mice, showing a prominent accumulation of Tregs in adenomas (338). Nevertheless, a comprehensive analysis of the myeloid cell compartment has not been reported thus far. In a spontaneous mouse model of breast
Discussion

cancer, Broz et al. have characterized the intratumoural myeloid cell compartment (339). Through their gating strategy they have identified two TAM subsets, MHCII<sup>hi</sup>CD11c<sup>lo</sup>CD11b<sup>hi</sup> TAMs and MHCII<sup>hi</sup>CD11c<sup>hi</sup>CD11b<sup>lo</sup> TAMs, which are likely the mammary tissue-resident macrophages and the tumour-restricted macrophages, respectively (52). In Franklin et al., both TAM subsets have been described in mammary tumours as well (52).

Our flow cytometry analysis revealed that colon tumours contain all the immune cell populations that are detected in the tumour-free colon LP, such as eosinophils (CD11b<sup>+</sup>F4/80<sup>int</sup>Ly6C<sup>-</sup>MHCII<sup>+</sup>), neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>); tissue-resident macrophages (CD11b<sup>+</sup>F4/80<sup>hi</sup>), the “waterfall configuration” with monocytes (CD11b<sup>+</sup>F4/80<sup>int</sup>Ly6C<sup>-</sup>MHCII<sup>+</sup>), and two monocyte-derived macrophage subsets (CD11b<sup>+</sup>F4/80<sup>int</sup>Ly6C<sup>+</sup>MHCII<sup>hi</sup> and CD11b<sup>+</sup>F4/80<sup>int</sup>Ly6C<sup>-</sup>MHCII<sup>hi</sup>, respectively), CD8<sup+</sup> and CD4<sup+</sup> T lymphocytes, Tregs, γ/δ T cells and B cells (Figure 3.5 and Figure 3.7).

However, the proportion of lymphoid and myeloid cell subsets in colon adenomas differs from that one in the LP. The LP contains mainly B lymphocytes, which make up 70% of CD45<sup>+</sup> cells, whereas adenomas are almost devoid of B cells (9% of CD45<sup>+</sup> cells) and all lymphocytes constitute only 21% of the leukocytes as the myeloid cells are more abundant (Figure 3.8). The analysis of the lymphoid cell compartment revealed a significantly higher prevalence of Tregs in the tumour than in the LP, as previously reported (338). Tumour-infiltrating CD8<sup+</sup> T cells are the least represented lymphocyte subset among those characterized. γ/δ T cells are about 6% of the leukocytes in the tumour, being more abundant than T cells and Tregs (Figure 3.8).

While eosinophils are the predominant LP myeloid cell subset, a massive accumulation of neutrophils and monocytes is observed in colon tumours, when compared to their counterparts in the LP (neutrophils: 40% of CD11b<sup>+</sup> myeloid cells versus 10%; monocytes: 7% of CD11b<sup>+</sup> myeloid cells versus 2%) (Figure 3.9). The infiltration of these two proinflammatory myeloid cells may play an important role in sustaining the chronic inflammation. We showed that the considerable influx of neutrophils in tumours occurs in the early phase of tumorigenesis and is maintained during the tumour progression (Figure 3.11). Notably, the CD11b<sup>+</sup>Ly6G<sup>+</sup> cell population may represent a continuum of developmental states, which could be granulocytic MDSCs. Interestingly, within the intratumoural F4/80<sup>hi</sup> macrophage subset we were able to clearly distinguish two subpopulations: F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> macrophages. On the contrary, in the LP mostly all F4/80<sup>hi</sup> tissue-resident macrophages are MHCII<sup>hi</sup>. Although MHCII<sup>lo</sup> cells are a minor population in the LP, they clearly accumulate in the tumour microenvironment, representing 20-30% of the tumour-resident F4/80<sup>hi</sup> macrophages (Figure 3.9).
The dynamic tumour microenvironment is also characterized by a stage-dependent milieu of cytokines and soluble factors, which are released by different types of infiltrating cells. The main source of MMPs is the stromal cells, such as fibroblasts, vascular cells and particularly immune cells (340). We could observe an increase in the abundance of both MMP9 and MMP12 along with the tumour progression, with different kinetics of accumulation (Figure 3.12). Ccl2 RNA levels markedly increase in advanced stages, when the tumour is 5-6 mm in diameter. Among the numerous functions, CCL2 has been identified as a crucial factor that facilitates the metastasis and dissemination of breast cancer cells and colon carcinoma cells, because it activates the CCR2+ endothelium to increase the vascular permeability and attracts the recruitment of inflammatory monocytes to promote the extravasation and seeding of metastatic tumour cells (212,341). CCL2 may also act in synergy with MMPs to promote the tumour invasiveness. We quantified the intratumoural levels of two M2-like immunosuppressive TAM markers, ARG1 and MARCO. ARG1 is known to indirectly inhibit T cell response by depriving T cells of l-arginine, which is necessary for their proliferation (342). MARCO – a pattern-recognition receptor of the class A scavenger receptor family – has been recently identified as overexpressed in the tumour microenvironment and linked to poor prognosis of human breast cancer (343). However, its role in cancer progression and the nature of the cells expressing this receptor are mostly unknown. We could also confirm the overexpression of both markers, suggesting an immunosuppressive environment in large adenomas. Colony-stimulating factors such as M-CSF (or CSF1), GM-CSF (or CSF2), granulocyte-CSF (G-CSF or CSF3) are also overexpressed in the colon tumour microenvironment. In preclinical mammary tumours, the overexpression of CSF1 (produced by macrophages, endothelial cells and fibroblasts) has been associated with recruitment and modulation of TAMs, and poor prognosis (255). CSF2 may have opposing effects: together with IFNγ likely polarizes macrophages towards a more cytotoxic and antigen-presenting phenotypes (344), in parallel it promotes tumour progression by enhancing tumour cell invasion (345). Moreover, CSF1/2/3 and VEGF promote myelopoiesis and contribute to the blockade of myeloid cell maturation that will ultimately generate MDSCs (188). Angiopoietin-2 (Angpt2), mostly produced by the vascular endothelium, accumulates along with the tumour progression. A trend of overexpression has also been detected for Hif1α and Vegfa. In conjunction with VEGF, ANGPT2 has been reported to induce vascular sprouting (346). Thus, proangiogenic factors are abundantly released in the microenvironment of developing tumours.
4.3 Turnover of macrophages in colon lamina propria

Given the high plasticity of the tumour microenvironment, we were particularly interested to understand the dynamics of TAM recruitment as well as the maintenance requirements in our model of colon cancer.

Under steady state conditions, it is well established that tissue-resident macrophages seed the tissues during fetal development, and in the adulthood are able to locally self-maintain through in situ proliferation without any contribution of haematopoietic progenitor cells from the adult BM (38). However, in tissues exposed to a low-grade inflammation, such as intestine and skin, or other triggers yet to be unraveled, the maintenance of the macrophage pool rigorously requires a continuous recruitment of BM-derived Ly6C\textsuperscript{hi} monocytes in a CCR2-dependent manner. Although embryo-derived macrophages are present in the intestine early in life, they do not persist in the adulthood to any substantial extent (58,59,108). This phenomenon of change in macrophage lineage can be explained with the “niche model” (106). The intestine is an “open” tissue, with no anatomical barriers that prevent the extravasation of circulating cells, and a low-grade inflammatory tone likely triggered by the continuous exposure to food antigens and microbiota. Thus, fetal resident macrophages are gradually outcompeted by monocyte-derived F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} for the available niches (59).

Herein, we have confirmed that in adult mice, under unperturbed conditions the vast majority of LP-resident macrophages are derived from circulating monocytes, which originate in the BM. Monocyte-derived macrophages comprise at least three subsets: F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} resident macrophages (fraction I) and the classical monocyte-derived intermediates F4/80\textsuperscript{int}Ly6C\textsuperscript{hi}MHCII\textsuperscript{hi} and F4/80\textsuperscript{int}Ly6C\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages (P2 and P3 in fraction II). P2 and P3 macrophages express very high levels of CCR2, followed by F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages (Figure 3.16). If the CCR2-CCL2 axis is perturbed, these cell subsets are drastically reduced in numbers (Figure 3.19). We were able to identify a sparse F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} macrophage population - most likely remnant fetal macrophages - that is retained in the adult colon, and does not rely on CCR2-CCL2 monocyte recruitment. Indeed, MHCII\textsuperscript{lo} macrophage numbers are unperturbed in adult Ccr2\textsuperscript{−/−} mice (Figure 3.19). In summary, in colon LP the tissue-resident macrophage population consists of two subpopulations – F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} and F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} – with distinct frequencies and maintenance requirements, however it is still not clear whether F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages are the ultimate differentiation step of the “monocyte-waterfall” from P3 macrophages or may derive directly from P2. Moreover, F4/80\textsuperscript{int}Ly6C\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages (P3) could eventually be considered as a mature macrophage subset, together with the two F4/80\textsuperscript{hi} subsets. Based on the cell markers
Ly6C, CX3CR1 and MHCII, other authors have already described the “P1–P4 waterfall” model of gut monocyte differentiation (57-59,102,347). Similarly to our analysis, Ly6C<sup>hi</sup>CX3CR1<sup>int</sup> circulating blood monocytes (P1) enter the tissue and progressively upregulate MHCII expression (P2 intermediates) and downregulate Ly6C levels as they become gut macrophages (P3 and P4). Based on CX3CR1 expression, MHCII<sup>hi</sup> macrophages can be further separated into a smaller CX3CR1<sup>int</sup> subpopulation (P3) and larger CX3CR1<sup>hi</sup> subpopulation (P4), with P4 cells being the most mature resident macrophages. While other authors considered P3 and P4 subsets as one macrophage population for their analyses (59,347), we have always maintained these two subsets separated as they may not overlap in phenotype and functions (347).

The fate mapping timepoint experiment with Kit<sup>MerCreMer/R26</sup> mice revealed that LP tissue-resident F4/80<sup>hi</sup> macrophages are characterized by a slower turnover rate compared to the other myeloid populations analyzed (Figure 3.22). In detail, BM-progenitors rapidly differentiate into monocytes and P2 intermediates, and it takes less than one week to generate the entire P2 subpopulation, whereas the differentiation step from P2 to P3 macrophages may require at least one more week. This could be due to a longer half-life of P3 macrophages as well as a longer timeframe for the differentiation into mature macrophages. F4/80<sup>hi</sup>MHCII<sup>hi</sup> macrophages become nearly completely YFP labelled almost half a year after tamoxifen administration. Even though it has been suggested that intestinal resident macrophages have an extremely short half-life of 3-5 weeks (107), we were able to demonstrate that if mature P3 macrophages are fully replaced within 3 weeks, for a fraction of F4/80<sup>hi</sup>MHCII<sup>hi</sup> macrophages it takes much more time, almost half a year. Furthermore, F4/80<sup>hi</sup>MHCII<sup>lo</sup> macrophages are minimally YFP labelled, suggesting that they are likely the fetal macrophages that are gradually diluted out after birth and almost disappear by the onset of adulthood. At 4 weeks post-tamoxifen treatment, 20% of the cells are YFP<sup>+</sup>; this indicates that monocytes can contribute to generate F4/80<sup>hi</sup>MHCII<sup>lo</sup> resident macrophages (Figure 3.22). The experiment with BM chimeric mice has confirmed these observations obtained with the fate mapping system. Recipient mice were irradiated, subsequently the BM was reconstituted with BM cells of donor mice. In the LP of chimeric mice, the vast majority of F4/80<sup>hi</sup>MHCII<sup>hi</sup> macrophages are derived from the donor BM, hence have BM origin. A considerable proportion of MHCII<sup>lo</sup> macrophages belongs to the recipient and is not replaced by cells derived from donor BM precursors (about 40% of the cells). (Figure 3.23).

Similarly to the gut, the skin is another anatomical site exposed to commensal microbes and under tonic inflammation (348). Consistent with the colon tissue-resident macrophages, dermal F4/80<sup>hi</sup>MHCII<sup>hi</sup> macrophages are BM-derived whereas
Discussion

F4/80hiMHCIIlo macrophages seed the fetal dermis and rapidly decline in the adulthood (40,51).

4.4 MHCIIlo macrophages disappear during ageing

In addition, we have characterized the kinetics of appearance/disappearance in the colon LP of these two distinct F4/80hi tissue-resident subpopulations with different BM-dependency. We performed an extensive characterization of the unperturbed LP at different timepoints of age, from E19.5 to 12 months, in order to “capture” the myeloid cell composition in the fetal colon as well as in the first days after birth and in the process of ageing. In contrast to Bain et al., who have described an increase of F4/80hiCD11bhi macrophage numbers and the disappearance of F4/80hiCD11blo macrophages as a function of age (59), our data showed that F4/80hi resident macrophages are preserved throughout the mouse lifespan, despite of some fluctuations in the numbers as well as the increase of CD11b surface expression, from CD11blo at E19.5 to CD11bhi from 3 weeks old. Interestingly, at E19.5 the fetal colon is mainly populated by tissue-resident F4/80hiMHCIIlo macrophages and monocytes. Two days after birth, likely due to the exposure to the external environment, neutrophils are massively recruited into the colonic mucosa and represent the most abundant myeloid cell type. One week after birth the LP is characterized by neutrophil clearance and a strong boost of both F4/80hiMHCIIhi and F4/80hiMHCIIlo macrophages. In the third week, the eosinophil population abundantly infiltrates the LP, making up 60% of the myeloid cells (Figure 3.13).

Among F4/80hi resident macrophages, MHCIIlo cells are the only resident macrophages in the fetal LP and can be detected at high frequency in young mice before the onset of the adulthood. However, as a function of age, they are gradually outcompeted by MHCIIhi macrophages (Figure 3.13). These data suggest that F4/80hiMHCIIlo macrophages are the embryonically derived resident macrophage population, which after birth is progressively replaced by F4/80hiMHCIIhi macrophages. The “monocyte waterfall” can be detected at E19.5; this indicates that the BM hematopoiesis has already started, thus explaining the presence of mature resident monocyte-derived macrophages immediately after birth. It remains to be established what causes the sudden boost of both F4/80hi macrophage subsets one week after birth. We may speculate that, due to certain triggers in the new mucosal environment, F4/80hiMHCIIlo embryo-derived macrophages undergo proliferation whereas F4/80hiMHCIIhi are massively generated through the “monocyte waterfall”.

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Bain et al. have also shown that the greatest change in macrophage numbers and monocyte recruitment occurred during the third week of life, and have proposed that this may be due to the establishment of the tonic commensal stimulation in the gut (59). At 3 weeks of age, they have observed a significant reduction of Ly6C^hi monocytes, Ly6C^+MHCII^+ macrophages and Ly6C^+MHCII^- mature macrophages in the colon of GF mice, when compared to that of CV mice. The defect in the numbers of monocytes and monocyte-derived macrophages might be due to an impaired recruitment of monocytes in absence of the microbiota. We have also analyzed the myeloid cell compartment in the colon of 8-week-old GF mice, when the gut microbiota is established and intestinal resident macrophages completely depend on the “monocyte waterfall” (58,59,108). In the colon of our GF mice we could appreciate a significant decrease in the proportion of neutrophils and monocytes, however P2-P3 and F4/80^hi macrophage numbers are not significantly reduced (Figure 3.14). Bain et al. have pointed out that no difference could be identified between 1-week-old GF mice and CV counterparts, in line with the idea that Ly6C^- macrophages start to be replenished by monocytes around the age of weaning (3 weeks old) (59). We speculate that perhaps at 3 weeks after birth the microbiota may impact considerably on the recruitment and differentiation of monocytes, however in adult mice (in this case 8-week-old mice) several other triggers may be involved. In line with our data, other studies have also provided opposing conclusions (103-105). It has been argued that the discrepancy in the results could be also due to a different composition of the commensal microbiome in animals from different sources (349-351), hence there might be specific bacterial species that can enhance or decrease the turnover rate of intestinal macrophages (352).

In conclusion, the adult colon contains at least 3 mature macrophage subsets. F4/80^-MHCII^- and F4/80^-MHCII^- macrophages constitute mostly the entire macrophage fraction and require the BM input to retain their cell pools with different turnover rates; the remnant fetal F4/80^-MHCII^- subset can be refilled by the BM, however this seems to be prevented under steady state conditions, perhaps because these cells are long-lived and the local niche might “force” the BM-precursors to differentiate into P3/F4/80^-MHCII^- macrophages. In contrast, a just published work is challenging the current understanding on gut macrophage maintenance (347). Following the “P1–P4 waterfall” and taking advantage of novel gut macrophage markers (TIM-4 and CD4), the authors have shown that the merged P3/P4 subset comprises three similarly sized subsets that are characterized by distinct replenishment rates from blood monocytes. TIM-4^-CD4^- macrophage subset has a high turnover from monocytes, while TIM-4^-CD4^- population has a slow turnover, and TIM-4^-CD4^- resident macrophages do not turn over from monocytes (347). This study claims that the fast
replenishment from monocytes does not characterize the entire gut macrophage population, but is limited to TIM-4^CD4^+ macrophages. Moreover, according to the authors, TIM-4^CD4^+ macrophages may be a previously unidentified resident macrophage population that can be maintained independently of adult blood monocytes (347).

**4.5 Intratumoural macrophages are independent from CCR2^+ monocytes**

Similar to the colon LP, we have investigated the ontogeny of TAMs in colon cancer. By definition, TAMs are all macrophages within a tumour microenvironment. Thus, F4/80^hi^MHCII^hi^ and F4/80^hi^MHCII^lo^, as well as P3 macrophages, constitute the TAM pool. In our DSS-accelerated tumour model, the analysis of tumours of different sizes revealed that along with the tumour progression the proportion of neutrophils, monocytes, P2-P3 cells and F4/80^hi^MHCII^hi^ macrophages does not change. The progressive increase in F4/80^hi^ tumour-resident macrophages is mainly due to the substantial accumulation of the MHCII^lo^ subpopulation. To strengthen this observation, we have dissected large tumours up to 5-6 mm in diameter in Apc^Min^/+ without any DSS treatment. Within the F4/80^hi^ subset, MHCII^lo^ macrophages outcompete MHCII^hi^ ones over time, and become the predominant macrophages in advanced tumour stages (Figure 3.15). We have thus speculated that F4/80^hi^MHCII^lo^ may play crucial functions to sustain the tumour growth.

Regarding the origins and mechanisms of TAM maintenance, only a few cancer types have been studied thus far. In breast cancer (52,197) and lung cancer (198,199) TAMs arise from Ly6C^hi^ circulating monocytes. For breast tumours, TAMs are completely dependent from the BM input, similarly to their counterparts in the healthy tissue (mammary tissue macrophages). In lung tumours, TAMs do not arise from the alveolar macrophages, which are known to be self-renewing embryo-derived macrophages, but from monocytes. In this case, heathy and neoplastic tissue contain mature macrophages of different origins, perhaps because the tumoural microenvironment may favour the engraftment and differentiation of monocytes. In addition, in mammary tumour models, TAM were shown to proliferate in situ, thus contributing to their accumulation (52,197).

Recent studies have proposed a mixture of TAMs with different origin in glioma (200) and pancreatic cancer (201): YS-derived and monocyte-derived TAMs. It is worth noting that in both healthy organs – brain and pancreas – tissue-resident macrophages derive from embryonic precursors and self-maintain in the adulthood. Thus, it is likely
that the tumour microenvironment may retain the original tissue-resident macrophage subset, and allows the engraftment of a new monocyte-derived subset.

In light of our previous findings on the LP-resident macrophages, we have characterized the origins and maintenance of macrophage subpopulations in colon tumours. Our analysis using Ccr2−/− mice has revealed that in the tumour F4/80hiMHCIIlo macrophages maintain a low expression of CCR2 and are not affected in their numbers when the CCR2-CCL2 axis is perturbed. This clearly suggests that MHCIIlo TAMs accumulate through other mechanisms. Interestingly, F4/80hiMHCIIhi TAMs downregulate CCR2 expression and lose BM-dependency (Figure 3.16 and 3.19). Of note, the tumour microenvironment does not favour monocyte differentiation to macrophages, as the “monocyte waterfall” seems to be blocked at the first stage of maturation, the Ly6Chi monocyte (Figure 3.9). This effect has also been described in the context of mammary tumours (52). We have speculated that it is unlikely that P2/P3 cells are the only source for F4/80hiMHCIIhi TAM maintenance. Our fate mapping system has shown that P3 TAMs are fully labelled, similarly to their LP counterparts, confirming that they differentiate from the monocytes. However, F4/80hiMHCIIhi macrophages have a lower percentage of labelling if compared to their LP counterparts (Figure 3.24a). We interpreted this result using the concept of the “niche model” adapted from (106) (Figure 4.2). In an early tumoural stage, F4/80hiMHCIIhi TAMs arise from the “monocyte waterfall”, hence maintain the BM-dependency that characterizes the intestinal macrophages under steady state. During the tumour progression changes in the environment may create a new balance of mechanisms that compete for the refilling and accumulation of the local TAM pool. Thus, F4/80hiMHCIIhi macrophages may lose their BM-dependency in late tumoural stages. In the case of F4/80hiMHCIIlo TAMs, the YFP labelling is comparable to that of the rare embryo-derived LP MHCIIlo macrophage counterpart. This suggests that the MHCIIlo TAM fraction retains the very low BM-dependency and contains macrophages of different ontogeny: YS/FL-derived and adult BM-derived TAMs. According to the “niche model”, we believe that when the tumour environment is not fully established, such as in an early tumoural stage, BM precursors may differentiate into MHCIIlo TAMs, whereas in subsequent stages this process unlikely occurs.
Figure 4.2: Proposed model of macrophage maintenance in early and late stage of colon cancer based on the three parameter “niche model”. In an early tumoural stage (upper panel), the tumour site contains the neovasculature and there are no anatomical barriers, hence macrophage niches are accessible via the blood stream. The niches are also available because TAMs have likely the same turnover of their LP counterparts, moreover the process of tumour expansion creates new niches. In absence of competing precursor, the niche refilling in the tumour occurs similarly to that of steady state and perturbed intestine: monocytes engraft and differentiate into macrophages. In a late tumoural stage (lower panel), niches are accessible, and available because the tumour continues to expand, however there might be two competing mechanisms of niche refilling: monocyte differentiation and local proliferation. This latter seems to be the most competitive mechanism. Concept taken from Guilliams M. and Scott C.L. Nat. Rev. Immunol. 17(7), 451-460 (2017).
4.6 Intratumoural resident macrophages expand by self-renewal

As described in breast cancer (52,197), we have also proposed that the accumulation of F4/80\textsuperscript{hi} TAMs may be driven by their in situ proliferation, rather than dependent on monocytic precursors. Our data clearly suggest that the tumour microenvironment of colon cancer triggers the self-renewal of F4/80\textsuperscript{hi} macrophages, which are actively cycling, unlike their macrophage counterparts in the colon LP (Figure 3.32). In a mature tumour microenvironment the local proliferation seems to be the most competitive mechanism that ensures TAM maintenance and, in particular, the accumulation of F4/80\textsuperscript{hi}/MHCII\textsuperscript{lo} TAMs (Figure 4.2). The high levels of CSF1 (Figure 3.29a), and likely other factors of the tumour microenvironment, may stimulate F4/80\textsuperscript{hi} macrophages to acquire the ability to self-renew within the tumour. It would be important now to further define the soluble factors and cells that shape this local environment that promotes self-renewal. Notably, the gained proliferative capability may represent an obstacle for therapeutic targeting of macrophages as well as for other immunotherapeutic approaches.

In Figure 4.3, we propose the mechanism of macrophage replenishment in both tumoural and healthy tissue in the colon. In the colon LP, newly steady state available niches are generated by the continuous turnover of MHCII\textsuperscript{hi} tissue-resident macrophages; hence circulating monocytes extravasate and engraft the unoccupied niches to differentiate into mature MHCII\textsuperscript{hi} macrophages. Embryonically derived MHCII\textsuperscript{lo} macrophages still persist in the adult LP, even though progressively diluted out and minimally replaced by monocyte-derived MHCII\textsuperscript{lo} macrophages.

As opposed to infections and other perturbations, whereby the resolution of inflammation restores a steady state condition, the tumour site is characterized by a non-resolving inflammation. In such chronically inflamed environment, TAM properties and mechanisms of refilling can be profoundly altered. Inflammation-triggered monocytes massively infiltrate the tumour, and in the early phase of tumour progression occupy the available inflamed niches to generate tumour-induced MHCII\textsuperscript{hi} TAMs. The fate of steady state tissue-resident MHCII\textsuperscript{hi} and MHCII\textsuperscript{lo} macrophages within the tumour is not clear. As suggested in a breast cancer model, tissue-resident macrophages of either embryonic or monocytic origin may undergo a change in phenotype/activation state during carcinogenesis (52). MHCII\textsuperscript{lo} TAMs are likely the fetal MHCII\textsuperscript{lo} resident macrophages, however they may also partially derive from monocytes. In late phases of tumorigenesis, the established tumour microenvironment confers to MHCII\textsuperscript{hi} and MHCII\textsuperscript{lo} TAMs the capability to self-renew. Hence, the potent in situ proliferation may drive the accumulation of TAMs, particularly MHCII\textsuperscript{lo} TAMs.
Figure 4.3: Proposed model of macrophage maintenance in colon tumour based on the three parameter “niche model”. In the peritumoural region, empty steady state macrophage niches (blue houses) are refilled (black arrow) by monocytes (pink cell) that give rise to mature MHCII\textsuperscript{hi} resident macrophages (light blue cell with pink nucleus). MHCII\textsuperscript{lo} resident macrophages (light blue cell with blue nucleus) still occupy some niches. In the tumour, empty inflamed macrophage niches (red houses) are refilled through the proliferation (curved black arrow) of MHCII\textsuperscript{hi} (red cell with red nucleus) and MHCII\textsuperscript{lo} (yellow cell with yellow nucleus) TAMs. Monocytes infiltrate the tumour site (red cells), and may be able to engraft (grey dashed arrow) the inflamed niche, especially in the early tumoural stage. Concept taken from Guilliams M. and Scott C.L. Nat. Rev. Immunol. 17(7), 451-460 (2017).

The fate mapping experiment in Ccr2\textsuperscript{-/-} mice has further corroborated these observations (Figure 3.27b). In the tumour, when ablating the CCR2-dependent source of monocytes, F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} and F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} TAMs are unperturbed in numbers (Figure 3.19) and display low labelling (20% of YFP\textsuperscript{+} cells). This confirms that the main source of niche refilling is local proliferation.

In another experiment we have purposely emptied the macrophage niches in LP and tumour by selectively ablating F4/80\textsuperscript{hi} macrophages. The almost complete depletion creates an “artificial” scenario, whereby the vast majority of the macrophage niches become vacant. In both tissue environments, LP- and tumour-resident F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} and F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} populations are completely YFP labelled as the other myeloid subsets. This suggests that the forced removal of one competitor – long-lived macrophages in the LP and self-maintaining macrophages in the tumour – enables circulating monocytes to engraft all emptied niches and differentiate into both MHCII\textsuperscript{hi} and MHCII\textsuperscript{lo} cells, in LP and tumour (Figure 3.28).
4.7 Anti-CSF1R antibody treatment depletes pro-tumoural macrophages

Subsequently, by neutralizing CSF1R we were able to investigate the tumour progression in absence of F4/80<sup>hi</sup> TAMs. We have demonstrated that intratumoural F4/80<sup>hi</sup> macrophages, and not monocytes and monocyte-derived P2/P3 macrophages, support cancer progression, suggesting this particular F4/80<sup>hi</sup> cell fraction as an attractive therapeutic target (Figure 3.33 and Figure 3.34). Another study, performed in pre-clinical tumour models, has also shown that the depletion of TAMs though anti-CSF1R monoclonal antibodies leads to a delay in tumour growth and reduces the number of spontaneously developed metastases (246). Nevertheless, in our tumour model the prolonged CSF1R blockade results in the depletion of both fractions of tumour- and LP-resident macrophages (Figure 3.30), and likely CSF1-dependent macrophages of other anatomical sites. This pan-macrophage therapeutic approach might cause systemic toxicities as it targets macrophages of other organs (226). Another therapeutic strategy aims to reprogram TAMs to exert anti-tumour functions, rather than ablating them. This result has been achieved, for instance, in pre-clinical models of glioma, whereby CSF1R blockade does not result in depletion of TAM numbers, but instead contributes to re-educate TAMs from having a protumour M2-like phenotype to an antitumour M1-like phenotype (227). However, in colon cancer the reprogramming cannot be obtain through CSF1R blockade.

Subsequently, we aimed to better characterize the in vivo functional phenotype of F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> TAMs, to assess whether they may be categorized as M1-like or M2-like TAMs. According to the “M1/M2 macrophage paradigm”, mainly defined through in vitro polarization experiments, proinflammatory M1-like TAMs display anti-tumoural properties, whereas alternatively activated M2-like TAMs are generally considered immune-suppressive and tumour-promoting cells. It is currently believed that both populations can be present within the tumour microenvironment, as well as TAMs with a functional phenotype in between M1-like and M2-like extremes (52,171). Moreover, TAMs with a certain specialization may occupy preferential areas of the tumour (162). Due to the peculiar environment of the gut (characterized by a tonic inflammation), the classification of intestinal tissue-resident macrophages does not follow the rigid M1–M2 dichotomy (121), as macrophages may have both M1 and M2 features (91,96). Similarly, there is an upregulation of the expression of M2-like markers such as Mmp2, Mmp9 and Mmp12 in F4/80<sup>hi</sup>MHCII<sup>hi</sup> and F4/80<sup>hi</sup>MHCII<sup>lo</sup> TAM subsets, suggesting an involvement in the ECM remodeling and tumour growth (Figure 3.37). However, both macrophage subsets are characterized by a glycolytic metabolism, which is typically a M1 signature. Moreover, they do not overexpress the M2 marker
Mrc1 (Cd206), along with other M2 markers, and even downregulate the expression of Cd163, compared to the F4/80\(^{hi}\) LP counterparts. Interestingly, MHCII\(^{lo}\) TAMs diverge from MHCII\(^{hi}\) TAMs and the LP macrophage subsets for the markedly high expression of M2 markers Arg1 and Marco (Figure 3.35). This latter under steady state has a restricted expression profile on tissue-resident macrophages in the lung, lymph nodes, spleen, and peritoneum (353,354). However, in the context of cancer it was identified as a gene overexpressed in the tumour microenvironment and linked to poor prognosis of human breast cancer (343). In line with our observation, a study in pre-clinical tumour models has recently shown that Marco was most highly expressed in MHCII\(^{lo}\) M2-like TAM subpopulation, which also expressed the M2 markers Cx3cr1, Arg1, and Retnla (Fizz1) but showed low expression of the M1-associated markers H2-ab1 (MhcII) and Nos2 (iNos). The anti-MARCO antibody treatment drastically skewed the TAM composition in the tumour microenvironment into a pro-inflammatory population, thereby rendering the tumour immunogenic and ultimately blocking the tumour growth and metastasis (354). As observed in other tumour models (209,355,356), F4/80\(^{hi}\)MHCII\(^{lo}\) macrophages, which become the predominant TAM subset during tumour development, likely represent a tumour-promoting macrophage subpopulation also in intestinal adenomas. Thus far, we could speculate that MHCII\(^{lo}\) TAMs contribute to shaping the tumour by directly depositing and/or remodelling the ECM (201). In parallel, they might gradually lose their capability to phagocytose and perform tumour antigen presentation to induce the adaptive immunity. In light of this, the anti-MARCO antibody may perhaps selectively target F4/80\(^{hi}\)MHCII\(^{lo}\) TAMs, leading to a more favorable outcome than that one obtained through CSF1R blockade.

### 4.8 Combination therapy: chemotherapy and anti-CSF1R antibody treatment

In light of the growing success of immunotherapy regimens across cancer types, there is currently a great interest in combining immunotherapeutic approaches with standard and novel treatments in order to exploit potential synergy. Standard cytoreductive therapies, such as chemotherapy and radiotherapy, have often adverse effects because they target not only neoplastic cells, but also healthy cells rapidly dividing. These include blood-producing cells, hair cells, and the cells of the mucus membranes of the mouth and throat area and of the digestive system. As a result, hair loss, anemia, nausea, vomiting, diarrhoea and infections in the mouth frequently are common short-term side-effects reducing patient quality of life. Type and severity of the side-effects depend also on the drug used and the treatment schedule. Here we show that a high-dose regimen of a 3-drug combination leads to a drastic reduction of the tumour load
but is extremely toxic (Figure 3.38). When the drug concentration is reduced to a suboptimal dosage that prevents mice from dying from the treatment cytotoxicity (Figure 3.39), the cytoreductive treatment becomes ineffective.

We have then hypothesized that the tolerable dose of chemotherapy in combination with macrophage-targeting CSF1R blockade may lead to a synergic effect. However, the combination has not significantly improved the therapeutic outcome obtained with anti-CSF1R antibody treatment alone. It is worth noting that CSF1R blockade causes a pan-macrophage ablation, including LP-resident macrophages that might promote tissue regeneration. In our cancer model, the severe depletion of LP-resident macrophages might be detrimental in conjunction with the chemotherapy, which is documented to induce, as side-effect, a change in gut permeability as well as mucositis (villus atrophy, shallow crypts, inflammation and ulceration) (357,358). We speculated that in Apc\(^{Min/+}\) mice chemotherapy-induced mucositis might likely correspond to a higher probability of tumour onset, especially when depleting tissue-repairing LP macrophages. We thus propose that the combination of chemotherapy with another M1 TAM-reprogramming approach may perhaps lead to a more successful outcome.

In conclusion, a better understanding of TAM ontogeny, mechanisms of maintenance and functional specialization in distinct tumours, such as the data presented here for colon cancer, will pave the way to the development of innovative macrophage-based therapeutic approaches, to be eventually combined with other antineoplastic therapies, including the promising immunotherapies that aim to restore the anti-tumoural T cell-mediated immunity (anti-PD-1, -PD-L1, or -CTLA4 drugs) (359,360).
5 CONCLUSION

Tissue-resident macrophages have been found to arise and seed the tissues during embryogenesis, and self-maintain locally in adulthood with minimal contribution from circulating monocytes. However, macrophages in the gut represent an exception to the rule. After birth, embryonically derived intestinal macrophages lose their proliferative capability and are outcompeted by monocyte-derived macrophages. Throughout the adult life intestinal macrophages rely on the BM input. Here, we have shown for the first time that in the unperturbed colon LP a minor F4/80\textsuperscript{hi}MHCII\textsubscript{lo} macrophage subpopulation still persists, although is progressively diluted out in the ageing tissue. These F4/80\textsuperscript{hi}MHCII\textsubscript{lo} macrophages are likely remnant long-lived fetal macrophages that have lost their self-renewing potential. They can be replaced by monocyte-derived counterparts, however this is a very slow process. As previously demonstrated, the vast majority of LP-resident macrophages consists of F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages that require a constant recruitment of circulating monocytic precursors via CCR2-CCL2 axis to retain their cell numbers. In colon adenomas, the tumour microenvironment reprograms both macrophage subsets to in situ proliferate. The vigorous self-renewal is sustained by CSF1 likely together with other factors of the tumour milieu. Moreover, F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} TAMs lose the BM dependency. Both TAM subsets, especially the MHCII\textsuperscript{lo} one, accumulate with tumour progression and promote tumour growth with their metabolic shift and upregulation of ARG1 and MMPs.

Figure 5.1: Summary of macrophage maintenance in healthy and tumoural tissues in colon. Left part: proposed scenario in colon LP. Right part: proposed scenario in colon adenoma. Legend: light blue cells: monocytes; blue cells with black nucleus: F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages; orange cells with black nucleus: F4/80\textsuperscript{hi}MHCII\textsuperscript{lo} macrophages. (Abbreviations: +++: high dependency; + and +/-: minimal dependency; curved arrow: local proliferation).
6 APPENDIX

6.1 Media, buffers and solutions

1X PBS
8 g NaCl
0.2 g KCl
1.44 g of Na$_2$HPO$_4$
0.24 g of KH$_2$PO$_4$
Adjust the pH to 7.2 with HCl
Add distilled water to a total volume of 1 litre

1% acid ethanol solution
Ethanol
1% HCl

1.5 % agarose gel
1X TAE buffer
1.5 % agarose
0.005% ethidium bromide

35% Percoll™ solution
157.5 ml Percoll™
325 ml 1X PBS
17.5 ml 10X PBS

40% Percoll™ solution
180 ml Percoll™
300 ml 1X PBS
20 ml 10X PBS

70% Percoll™ solution
315 ml Percoll™
150 ml 1X PBS
35 ml 10X PBS

50X TAE buffer (Tris/acetate/EDTA)
242 g Tris base
57.1 ml glacial acetic acid
37.2 g Na$_2$EDTA•2H$_2$O
Distilled H$_2$O to 1 litre

Anti-CSF1R antibody
1X sterile PBS
Anti-CSF1R antibody (Clone AFS98, BioXCell, West Lebanon, NH, USA)
Anti-CSF1R antibody intraperitoneal injection (400 μg) using 1-ml insulin syringe. In control group, intraperitoneal injection of rat IgG2a isotype control (Biolegend).

Blocking buffer for cryosections
1X PBS
10% FCS
0.1% Sodium Azide from 10% (w/v) sodium azide

**Digestion medium**
Iscove’s Modified Dulbecco’s Medium (IMDM)
2% FCS

**Diphtheria Toxin (DT)**
1x sterile PBS
1% mouse serum
2 ng/μl DT
DT intraperitoneal injection (20 ng/g body weight) using 1-mL UltraFine™ insulin syringe (BD, Franklin Lakes, NJ, USA)

**Fc Block**
1X PBS
2% FCS
1:350 Home-made anti-mouse CD16/CD32 (Fc Block) 3.5 mg/ml

**Organ fixation buffer**
1X PBS
4% paraformaldehyde
pH adjusted to 7.4

**PBS 2%**
1X PBS
2% FCS

**Scott’s tap water substitute**
For 500 ml:
1 g sodium bicarbonate
10 g magnesium sulphate
Distilled H₂O to 500 ml

**SNET Buffer**
20 mM Tris-HCl (pH 8.0)
5 mM EDTA (pH 8.0)
400 mM NaCl
1% (w/v) SDS
Distilled H₂O to 1 liter

**Tamoxifen**
Corn oil (C8267; Sigma-Aldrich, St. Louis, MO, USA)
4 mg/injection Tamoxifen (T5648; Sigma-Aldrich)
Tamoxifen administration using a feeding needle in Omnifix®-F Luer Solo 1ml-syringe (B. Braun, Melsungen, Germany)

**TE Buffer 10X**
100 mM Tris (pH 8.0)
100 mM EDTA (pH 8.0)
Distilled H₂O to 250 ml
6.2 Reagents, chemicals and kits

Acetic acid (glacial) Merck KGaA, Darmstadt, Germany
Acetone Fisher Scientific UK, Loughborough, UK
Agarose 1st BASE Pte Ltd, Singapore
Ammonium chloride Sigma, St. Louis, MO, USA
Arcturus™ PicoPure™ RNA Isolation Kit (Cat. No. KIT0214) Thermo Fisher Scientific, Waltham, MA, USA
BD Cytofix/Cytoperm™ Kit BD, San Diego, CA, USA
Collagenase D Roche, Basel, Switzerland
Corn oil Sigma, St. Louis, MO, USA
DAPI Thermo Fisher Scientific, Waltham, MA, USA
(D4',6-diamidino-2-phenylindole) (Invitrogen™)
DNase I Life Technologies, Carlsbad, CA, USA
dNTP Set (4 x 25 μmol) Thermo Fisher Scientific, Waltham, MA, USA
DPX Mountant for histology Sigma, St. Louis, MO, USA
DSS MPBiomedical, Santa Ana, CA, USA
Diptheria Toxin, from Sigma, St. Louis, MO, USA
Corynebacterium diphtheriae EDTA Disodium Salt, Dihydrate USB Corporation, Cleveland, OH, USA
Eosin Y 5 wt. % solution in water Sigma, St. Louis, MO, USA
ERCC RNA Spike in Controls (Cat. No. 4456740) Ambion® Thermo Fisher Scientific, Waltham, MA, USA
Ethanol Merck KGaA, Darmstadt, Germany
Ethidium Bromide solution Bio-Rad, Hercules, CA, USA
FAST 2x qPCR Master mix PrecisionFAST-SY, Primerdesign Ltd, Cambridge, UK
FCS Chemicon International, Temecula, CA, USA
Fluorescence mounting medium Dako, Golstrup, Denmark
Fluorouracil (50 mg/ml) Teva Pharmaceutical Industries Ltd, Petach Tikva, Israel
Foxp3 Fixation/Permeabilization Concentrate and Diluent eBioscience, San Diego, CA, USA
Foxp3 Permeabilization buffer 10x eBioscience, San Diego, CA, USA
<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneRuler 1kb DNA ladder</td>
<td>Fermentas, Waltham, MA, USA</td>
</tr>
<tr>
<td>Giemsa stain, modified</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>GoTaq Flexi DNA Polymerase</td>
<td>Promega, Fitchburg, WI, USA</td>
</tr>
<tr>
<td>Hematoxylin solution modified acc. to Gill for microscopy</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Hydrochloric acid fuming 37% (HCl)</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Illumina Nextera XT DNA Sample Preparation kit (Cat. No. FC-131–1024)</td>
<td>Illumina, San Diego, CA, USA</td>
</tr>
<tr>
<td>IMDM</td>
<td>Gibco, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Irinotecan (20mg/ml)</td>
<td>Hospira, Lake Forest, IL, USA</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck KgA, Darmstadt, Germany</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase</td>
<td>Promega, Fitchburg, WI, USA</td>
</tr>
<tr>
<td>M-MLV RT 5X Reaction Buffer</td>
<td>Promega, Fitchburg, WI, USA</td>
</tr>
<tr>
<td>Nuclease-free water (Invitrogen™)</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>OligodT primers</td>
<td>Promega, Fitchburg, WI, USA</td>
</tr>
<tr>
<td>Ovation® Pico WTA System Kit (Part No. 3302)</td>
<td>NuGEN Technologies, San Carlos, CA, USA V2</td>
</tr>
<tr>
<td>Oxaliplatin (5mg/ml)</td>
<td>Hospira, Lake Forest, IL, USA</td>
</tr>
<tr>
<td>Paraplast Plus (Paraffin wax)</td>
<td>Leica Biosystems, Wetzlar, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma, St. Louis, MO, USA</td>
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<tr>
<td>PBS 10X</td>
<td>Gibco, Grand Island, NY, USA</td>
</tr>
<tr>
<td>Percoll™</td>
<td>GE Healthcare, Uppsala, Sweden</td>
</tr>
<tr>
<td>Perkin Elmer Labchip (Cat. No. CLS760672)</td>
<td>Perkin Elmer, Waltham, MA, USA</td>
</tr>
<tr>
<td>Phosphate-Buffered Saline (10X) pH 7.4 (Invitrogen™)</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Merck KgA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Potassium phosphate monobasic (H2KO4P)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Promega, Fitchburg, WI, USA</td>
</tr>
<tr>
<td>Quant-iTTM RiboGreen® RNA Reagent and Kit</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
</tbody>
</table>
RiboLock RNase Inhibitor
Thermo Fisher Scientific, Waltham, MA, USA

RNaseZAP™
Sigma, St. Louis, MO, USA

RNAsimple Total RNA Kit
Tiangen Biotech Co. Ltd, Beijing, China

Sodium Azide
Sigma, St. Louis, MO, USA

Sodium chloride (NaCl)
Merck KgaA, Darmstadt, Germany

Sodium Dodecil Sulfate (SDS)
Fluka™, Honeywell Research Chemicals Shanghai, China

Sodium phosphate dibasic dihydrate (HNa$_2$O$_4$P•2H$_2$O)
Sigma, St. Louis, MO, USA

Tamoxifen
Sigma, St. Louis, MO, USA

First-strand buffer 5X
Invitrogen, Carlsbad, CA, USA

Tissue-Tek OCT compound
Sakura Finetek, Torrance, CA, USA

Tris
Affymetrix USB, Cleveland, OH, USA

TRIzol™ Reagent
Life Technologies, Carlsbad, CA, USA

Xylene
Sigma, St. Louis, MO, USA

Table 7: Commercial antibodies

<table>
<thead>
<tr>
<th>Anti-mouse antibody</th>
<th>Clone</th>
<th>Company</th>
<th>Label</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG1</td>
<td>-</td>
<td>R&amp;D System</td>
<td>FITC</td>
<td>FACS</td>
</tr>
<tr>
<td>CCR2</td>
<td>-</td>
<td>R&amp;D System</td>
<td>APC</td>
<td>FACS</td>
</tr>
<tr>
<td>CD45.2</td>
<td>104</td>
<td>eBioscience</td>
<td>PE-Cy7</td>
<td>FACS</td>
</tr>
<tr>
<td>CD45.1</td>
<td>A20</td>
<td>BioLegend</td>
<td>FITC</td>
<td>FACS</td>
</tr>
<tr>
<td>CD45</td>
<td>30-F11</td>
<td>BioLegend</td>
<td>APC-Cy7</td>
<td>FACS</td>
</tr>
<tr>
<td>CD3</td>
<td>17A2</td>
<td>BD</td>
<td>BuV737</td>
<td>FACS</td>
</tr>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>eBioscience</td>
<td>APC</td>
<td>FACS</td>
</tr>
<tr>
<td>CD45R (B220)</td>
<td>RA3-6B2</td>
<td>BioLegend</td>
<td>PE-Cy7</td>
<td>FACS</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>BioLegend</td>
<td>APC-Cy7</td>
<td>FACS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BuV395</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>GK1.5</td>
<td>BD eBioscience</td>
<td>APC-Cy7</td>
<td>FACS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FITC</td>
<td></td>
</tr>
<tr>
<td>CD8α</td>
<td>53-6.7</td>
<td>BD</td>
<td>BuV395</td>
<td>FACS</td>
</tr>
<tr>
<td>CD326 (EpCAM)</td>
<td>G8.8</td>
<td>eBioscience</td>
<td>APC</td>
<td>Tissue</td>
</tr>
<tr>
<td>F4/80</td>
<td>BM8</td>
<td>eBioscience</td>
<td>PE</td>
<td>FACS</td>
</tr>
<tr>
<td>Foxp3</td>
<td>FJK-16s</td>
<td>eBioscience</td>
<td>PE</td>
<td>APC</td>
</tr>
<tr>
<td>Ki67</td>
<td>SolA15</td>
<td>Miltenyi Biotech</td>
<td>FITC (Vio&lt;sup&gt;®&lt;/sup&gt;515)</td>
<td>FACS</td>
</tr>
<tr>
<td>SiglecF</td>
<td>551</td>
<td>BioLegend</td>
<td>APC</td>
<td>FACS</td>
</tr>
</tbody>
</table>
### Appendix

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Label</th>
<th>Dilution in PBS 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly6C</td>
<td>BioLegend</td>
<td>BV605 PerCP-Cy5.5 FACS</td>
</tr>
<tr>
<td>Ly6G</td>
<td>1A8 BD</td>
<td>BuV395 FITC FACS</td>
</tr>
<tr>
<td>MHC class II (I-a-I-E)</td>
<td>M5/114.15.2 eBioscience</td>
<td>eFluor®450 FACS</td>
</tr>
<tr>
<td>B-catenin</td>
<td>15B8 eBioscience</td>
<td>FITC Tissue</td>
</tr>
<tr>
<td>γ/δ TCR</td>
<td>GL-3 eBioscience</td>
<td>APC FACS</td>
</tr>
</tbody>
</table>

**Table 8:** Antibody dilutions in flow cytometry

<table>
<thead>
<tr>
<th>Anti-mouse antibody</th>
<th>Label</th>
<th>Dilution in PBS 2% (or perm. buffer for intracellular markers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG1</td>
<td>FITC</td>
<td>1:20</td>
</tr>
<tr>
<td>CCR2</td>
<td>APC</td>
<td>1:20</td>
</tr>
<tr>
<td>CD45.2</td>
<td>PE-Cy7</td>
<td>1:600</td>
</tr>
<tr>
<td>CD45.1</td>
<td>FITC</td>
<td>1:600</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-Cy7</td>
<td>1:600</td>
</tr>
<tr>
<td>CD3</td>
<td>BuV737</td>
<td>1:600</td>
</tr>
<tr>
<td>CD11c</td>
<td>APC</td>
<td>1:600</td>
</tr>
<tr>
<td>CD11c</td>
<td>PE-Cy7</td>
<td>1:600</td>
</tr>
<tr>
<td>CD45R (B220)</td>
<td>BV605</td>
<td>1:600</td>
</tr>
<tr>
<td>CD11b</td>
<td>APC-Cy7</td>
<td>BuV395</td>
</tr>
<tr>
<td>CD4</td>
<td>APC-Cy7</td>
<td>FITC</td>
</tr>
<tr>
<td>CD8α</td>
<td>BuV395</td>
<td>1:600</td>
</tr>
<tr>
<td>F4/80</td>
<td>PE</td>
<td>1:600</td>
</tr>
<tr>
<td>Foxp3</td>
<td>PE</td>
<td>1:600</td>
</tr>
<tr>
<td>Foxp3</td>
<td>APC</td>
<td>1:600</td>
</tr>
<tr>
<td>Ki67</td>
<td>FITC</td>
<td>1:40</td>
</tr>
<tr>
<td>SiglecF</td>
<td>APC</td>
<td>1:600</td>
</tr>
<tr>
<td>Ly6C</td>
<td>BV605 PerCP-Cy5.5</td>
<td>1:600 1:1000</td>
</tr>
<tr>
<td>Ly6G</td>
<td>BuV395</td>
<td>FITC</td>
</tr>
<tr>
<td>MHC class II (I-a-I-E)</td>
<td>eFluor®450</td>
<td>FITC</td>
</tr>
<tr>
<td>γ/δ TCR</td>
<td>APC</td>
<td>1:600</td>
</tr>
</tbody>
</table>

**Table 9:** Antibody dilutions in immunohistochemistry

<table>
<thead>
<tr>
<th>Anti-mouse antibody</th>
<th>Label</th>
<th>Dilution in Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD326 (EpCAM)</td>
<td>APC</td>
<td>1:300</td>
</tr>
<tr>
<td>B-catenin</td>
<td>FITC</td>
<td>1:300</td>
</tr>
</tbody>
</table>
### Table 10: Primer sequences for PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer sequence 5' → 3'</th>
<th>Reverse primer sequence 5' → 3'</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apc&lt;sup&gt;Min+/−&lt;/sup&gt;</em></td>
<td>5'-ttc tga gaa aga cag aag tta-3'</td>
<td>5'-ttc cac ttt gcc ata agg c-3'</td>
<td>Heterozygote: 331 bp WT: no amplicon</td>
</tr>
<tr>
<td>Internal positive control</td>
<td>5'-caa atg tgt gtt gtc tgg tg-3'</td>
<td>5'-gtc agt cga gtt cac agt tt-3'</td>
<td>200 bp</td>
</tr>
<tr>
<td><em>Ccr2&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td><strong>Common</strong>: 5'-cca cag aat cca agg aaa tgg-3'</td>
<td><strong>WT</strong>: 5'-aca gca tga ata gcc aag-3'</td>
<td>Mutant: ~390 bp Heterozygote: ~390 bp + 494 bp WT: 494 bp</td>
</tr>
<tr>
<td><em>LoxP-STOP-LoxP</em></td>
<td><strong>WT</strong>: 5'-aaa gtc gct ctg agt tgt tat-3'</td>
<td><strong>WT</strong>: 5'-ggc gga gaa atc gat-3'</td>
<td>Mutant <em>LSL</em>: ~300 bp Heterozygote <em>LSL</em>: ~300 bp + ~550 bp WT <em>LSL</em>: ~550 bp</td>
</tr>
<tr>
<td><em>Kit</em></td>
<td><strong>Common</strong>: 5'-ggc acc ata gcc aag agt tgg tc-3'</td>
<td><strong>Mutant</strong>: 5'-ctt ctg gcc tgt agc atg-3'</td>
<td>Mutant <em>Kit</em>: ~300 bp Heterozygote <em>Kit</em>: ~300 bp and ~800 bp WT <em>Kit</em>: ~800 bp</td>
</tr>
<tr>
<td><em>Csf2r&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td><strong>Mutant</strong>: 5'-ata tgc ctg agc tgg gcc g-3'</td>
<td><strong>Common</strong>: 5'-gaa gcc tca atg ttc tga tgg gat-3'</td>
<td>Mutant: 648 bp Heterozygote: 300 bp and 648 bp WT: 300 bp</td>
</tr>
<tr>
<td><em>hDTR</em></td>
<td>5'-ttg ggt gag gac aac aac atg gcc-3'</td>
<td>5'-tac agc tct gtc ttc ggc tca-3'</td>
<td>698 bp</td>
</tr>
<tr>
<td><em>Cx3cr1-gfp</em></td>
<td><strong>WT</strong>: 5'-gtc ttc acg ttc ggt ctg g-3'</td>
<td><strong>Common</strong>: 5'-ccc aga cac tgg tgt ttc ct-3'</td>
<td>Mutant: ~500 bp Heterozygote: 410 bp and ~500 bp WT: 410 bp</td>
</tr>
</tbody>
</table>

### Table 11: Primer sequences for real-time qPCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer sequence 5' → 3'</th>
<th>Reverse primer sequence 5' → 3'</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Angpt2</em></td>
<td>5'-ctc acc acc agt ggc atc ta-3'</td>
<td>5'-ccc acc gtc tgc atc gaa c-3'</td>
<td>80 nt</td>
</tr>
<tr>
<td><em>Arg1</em></td>
<td>5'-gaa tct gca tgg gcc acc-3'</td>
<td>5'-gaa ttc tga tgc gca c-3'</td>
<td>73 nt</td>
</tr>
<tr>
<td><em>Ass1</em></td>
<td>5'-tgg cca gga aag cag act ac-3'</td>
<td>5'-agc ctt tgc tga aat c-3'</td>
<td>74 nt</td>
</tr>
<tr>
<td><em>Ccl2</em></td>
<td>5'-ctg cca ggt ggc tca-3'</td>
<td>5'-gat cat ctt gct gta gaa c-3'</td>
<td>76 nt</td>
</tr>
<tr>
<td><em>Ccna2</em></td>
<td>5'-tgc act acc atc ttt ctt agg-3'</td>
<td>5'-tgtaat act gct tca aat tcc-3'</td>
<td>96 nt</td>
</tr>
<tr>
<td><em>Cdc8a</em></td>
<td>5'-gcc ata aga atc ttc ctg atc-3'</td>
<td>5'-ttc tga tat gga ctg gtt tct c-3'</td>
<td>74 nt</td>
</tr>
<tr>
<td><em>Cdc20</em></td>
<td>5'-aca tca agg cgc tgt cca g-3'</td>
<td>5'-ctc aga cgt tca aag c-3'</td>
<td>110 nt</td>
</tr>
<tr>
<td><em>Cdc25c</em></td>
<td>5'-gga acc acc cgg atc tga a-3'</td>
<td>5'-act ttc cag gca aag cag c-3'</td>
<td>63 nt</td>
</tr>
</tbody>
</table>
### Table 12: PCR and real-time qPCR programmes

<table>
<thead>
<tr>
<th>Genotyping</th>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotyping <em>Apc</em>&lt;sup&gt;Min/+&lt;/sup&gt;</td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>95°C 0:30; 55°C 0:30; 72°C 0:45 (x35)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>72°C 3:00</td>
<td>4.</td>
<td>16°C ∞</td>
</tr>
<tr>
<td>Genotyping <em>Ccr2</em>&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>95°C 0:30; 61°C 0:30; 72°C 1:00 (x35)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>72°C 3:00</td>
<td>4.</td>
<td>16°C ∞</td>
</tr>
<tr>
<td>Genotyping <em>Kit</em>&lt;sup&gt;MerCreMer/R26 (LoxP-STOP-LoxP locus and Kit locus)&lt;/sup&gt;</td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>95°C 0:30; 55°C 0:30; 72°C 0:45 (x35)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>72°C 3:00</td>
<td>4.</td>
<td>16°C ∞</td>
</tr>
<tr>
<td>Genotyping <em>Csf2r</em>&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>95°C 0:30; 62°C 0:30; 72°C 0:45 (x35)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>72°C 2:00</td>
<td>4.</td>
<td>16°C ∞</td>
</tr>
<tr>
<td>Genotyping <em>hDTR</em></td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>95°C 0:30; 61°C 0:30; 72°C 1:00 (x35)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>72°C 3:00</td>
<td>4.</td>
<td>16°C ∞</td>
</tr>
<tr>
<td>Genotyping <em>Cx3cr1-gfp</em></td>
<td>1.</td>
<td>95°C 2:00</td>
<td>2.</td>
<td>94°C 0:20; 65°C 0:15 (-0.5°C per cycle decrease); 68°C 0:10 (x10)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>3.</td>
<td>94°C 0:15; 60°C 0:15; 72°C 0:10 (x28)</td>
<td>4.</td>
<td>72°C 2:00</td>
</tr>
<tr>
<td>Genotyping</td>
<td>5.</td>
<td>16°C ∞</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3 Equipment

| Real-time qPCR | 1. 95°C 10:00  
|               | 2. 95°C 0:10; 60°C 0:30 (x40)  
|               | 3. 95°C 0:15  
|               | 4. 55°C 0:15  
|               | 5. 95°C 0:15  |

100 μm Falcon™ Cell Strainer | BD Biosciences, San Jose, CA, USA
5-laser Fortessa™ X-20 cell analyzer | BD Biosciences, San Jose, CA, USA
Agilent Bioanalyser | Agilent, Santa Clara, CA, USA
BioBeam gamma irradiation device | Gamma Service Ltd., NY, USA
Eco™ plate48 | Cole-Parmer, Stone, Staffordshire, UK
Eco™ Real-Time PCR system | Illumina, San Diego, CA, USA
Embedding cassettes with removable lid | Practical Mediscience, Singapore, Singapore
Eppendorf® Centrifuge 5810R | Sigma, St. Louis, MO, USA
Eppendorf® Thermomixer compact | Sigma, St. Louis, MO, USA
FACSAria™ cell sorter | BD Biosciences, San Jose, CA, USA
Illumina HiSeq 2500 system | Illumina, San Diego, CA, USA
Incubator shaker Innova 4200 | Edison, NJ, USA
Leica CM3050 S – Cryostat | Leica Microsystems, Wetzlar, Germany
Leica EG1150 C – Embedding | Leica Microsystems, Wetzlar, Germany
Leica EG1150 H – Embedding | Leica Microsystems, Wetzlar, Germany
Leica CM1950 - Microtome | Leica Microsystems, Wetzlar, Germany
Leica RM2265 – Microtome | Leica Microsystems, Wetzlar, Germany
Leica 818 high profile microtome blades | Leica Microsystems, Wetzlar, Germany
Microscope Nikon Eclipse 80i | Nikon Instruments, Melville, NY, USA
Microscope VFM coverslips | CellPath Ltd, Newton UK
Microscope coated glass slides | Thermo Scientific, Loughborough, UK
Microscope glass slides | Continental Lab Products (CLP), San Diego, CA, USA
pHmeter | Mettler-Toledo Ltd., Port Melbourne, Australia
Shandon™ Cytoclip
Shandon™ Cytofunnel™
Shandon™ filter cards
T Professional TRIO Thermocycler
Thermo Scientific™ Cytospin™ 4 Cytocentrifuge
Thermo Scientific™ NanoDrop™ OneC Spectrophotometer
X360 high speed homogenizer drive with P1500 variable speed control

Thermo Scientific, Loughborough, UK
Thermo Scientific, Loughborough, UK
Thermo Scientific, Loughborough, UK
Biometra GmbH, Goettingen, Germany
Thermo Scientific, Loughborough, UK
Fisher Scientific UK, Loughborough, UK
CAT Scientific, Paso Robles, CA, USA
7 AUTHOR’S PUBLICATIONS


8 POSTERS AND INVITED ORAL PRESENTATIONS

2018:
5th European Congress of Immunology, Amsterdam, September 2018.

2017:
Oral presentation: “Tumor microenvironment supports the self-maintenance of intestinal tissue-resident macrophages.” Soncin I, Sheng J, Chen Q, Karjalainen K, Ruedl C.

Poster presentation: “Tumor microenvironment supports the self-maintenance of intestinal tissue-resident macrophages.” Soncin I, Sheng J, Chen Q, Karjalainen K, Ruedl C.

6th Winter School in Advanced Immunology IFReC-SlgN, Singapore, January 2017.
Oral and poster presentation: “Tumor microenvironment supports the self-maintenance of intestinal tissue-resident macrophages.” Soncin I, Sheng J, Chen Q, Ruedl C.

2016:
5th Advanced Singaporean Immunology PhD Student Retreat, Singapore, September 2016.

International Congress of Immunology, Melbourne, Australia, August 2016.
Poster presentation: “Role of intestinal CD103⁺CD11b⁻ dendritic cells in colorectal cancer.” Soncin I, Sheng J, Ruedl C.

8th International Singapore Symposium of Immunology (Singapore), May 2016.
Poster presentation: “Role of intestinal myeloid cell subsets in colorectal cancer.” Soncin I, Chen Q, Foo HR, Sheng J, Ruedl C.

2015:
6th Congress of the FIMSA, Singapore, June 2015.
9 AWARDS

2016: Conference Travel Award, Singapore Society of Immunology.

2018: “Women in Science” conference grant, College of Science, Nanyang Technological University.
10 REFERENCES

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