UNIVERSIDADE DE LISBOA

Faculdade de Medicina

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Supervisor: Prof. Karine Marie Serre, PhD.

Dissertação especialmente elaborada para obtenção do grau de

Mestre em Oncobiologia, área de especialização Investigação em Oncobiologia

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LIST OF ABBREVIATIONS

2-DG – 2-Deoxyglucose
ACT – Adoptive Cell Therapy
APC – Antigen-Presenting Cell
ATP – Adenosine TriPhosphate
BM – Bone Marrow
BMDM – Bone Marrow-Derived Macrophages
CD – Cluster of Differentiation
cDMEM – complete DMEM
cRPMI – complete RPMI
CAR – Chimeric Antigen Receptor
CCL – CC Chemokine Ligand
CSF – Colony Stimulating Factor
CTLA4 – Cytotoxic T Lymphocyte-Associated protein 4
DC – Dendritic Cell
DMEM – Dulbecco's Modified Eagle Medium
EM – Energetic Metabolism
FAD – Flavin Adenine Dinucleotide
$FADH_2$ – reduced FAD
FAO – Fatty Acid Oxidation
FDA – Food and Drug Administration
FCS – Fetal Calf Serum
ICB – Immune Checkpoint Blockade
IFN – Interferon
IL – Interleukin
IMM – Instituto de Medicina Molecular
iNOS – Inducible Nitric Oxide Synthase
LPS – Lipopolysaccharide
mAb – monoclonal Antibody
M-CSF – Macrophage-CSF
MCT – Myeloid-Cell Treatment
MDSC – Myeloid Derived Suppressor Cell
MHC – Major Histocompatibility Complex
NAD – Nicotinamide Adenine Dinucleotide
NAD(P)H – reduced NAD
NK – Natural Killer
NO – Nitric Oxide
NT – Not Treated
OXPHOS – Oxidative Phosphorylation
P/S – penicillin/streptomycin
PD – Programmed Death receptor
PD-L – Programmed Death receptor-Ligand
PI – Propidium Iodide
PRR – Patter Recognition Receptor
RBC – Red Blood Cell
RPMI – Roswell Park Memorial Institute
RT – Room Temperature
cRPMI – complete RPMI
SN – Supernatant
TAMs – Tumour Associated Macrophages
TCA – Tricarboxylic Acid
TCR – T Cell Receptor
TGF – Transforming Growth Factor

TIL – Tumour-Infiltration Lymphocyte
TNBC – Triple Negative Breast Cancer
TLR – Toll Like Receptor
TME – Tumour Microenvironment
TNF – Tumour Necrosis Factor
WT – Wild-type

ABSTRACT

The tumour microenvironment (TME) is a heterogeneous ecosystem populated by myeloid and lymphoid immune cells that have the natural capacity to patrol and eliminate nascent tumours, mainly through the cytotoxic properties of T cells. However, immune cells often fail to control tumour growth due to escape mechanisms induced by tumour cells themselves that halt antitumour immunity and ultimately prevent tumour eradication. These include immunosuppressive soluble factors, deleterious cell to cell interactions and metabolic competition for nutrients. Among the cellular participants of the TME modulated by tumour cells, the myeloid compartment has a prominent role as they can exert antitumoural or protumoural functions according to the surrounding environmental cues. With this is mind, we believe there has been a disproportionate attention on protumour over antitumour myeloid cell functions, and that more efforts should be put on understanding how to enhance the protective activity of myeloid cells in the context of cancer.

In this thesis, we took advantage of myeloid cell inherent capacity to respond to maturing agents, such as TLR ligands and co-stimulatory agonists, to study their antitumour potential. Using the E0771 TNBC orthotopic mouse model, we observed that the injection of TLR3 ligand plus an anti-CD40 agonistic mAb (Myeloid-Cell Treatment; MCT hereafter) was able to induce tumour regression in the vast majority of treated mice. The prevention of this regression by clodronate-encapsulated liposomes suggested a key antitumour role for macrophages. Furthermore, MCT-activated tumour-associated macrophages (TAMs) overexpressed TNFα and iNOS, which are known tumoricidal molecules, when compared to the respective controls. Taking advantage of *in vitro* bone marrow-derived macrophages (BMDMs) to dissect the contributions of each molecule, we first observed that the supernatant derived from MCT-stimulated BMDMs increased E0771 cell death, demonstrating their antitumour properties. BMDMs also presented increased expression of TNFα and iNOS. However, *in vivo* and *in vitro* studies with anti-TNFα blocking antibodies suggested a neglectable role of TNFα in MCT-induced antitumour immunity. Together with *in vivo* experiments with an TNFα-resistant E0771 clone, we excluded TNFα as the soluble mediator of antitumour MCT-TAMs in this model. Finally, iNOS expression in MCT-BMDMs was found critical for the decreased tumour cell viability *in vitro*, thus suggesting MCT programmes TAMs with iNOS-dependent antitumour properties.

Given that cellular metabolism can dictate the functional outcome of immune cells, we then investigated the metabolic requirements of MCT BMDMs. To our surprise, we found that MCT BMDMs differed from pro-inflammatory/antitumoural *in*

vitro-differentiated M1 macrophages, which were markedly glycolytic, as they instead displayed a mitochondrial dependency that was similar to anti-inflammatory/protumoural M2 macrophages, which reflected on comparable ATP contents in MCT and M2 BMDMs. These data contradicted previous reports that linked glucose oxidation with antitumour functions, and led us to investigate the metabolic intermediates that supported MCT-BMDMs mitochondrial metabolism. We observed MCT-BMDMs had similar glucose uptake compared to M1, and distinct lipid content when compared to M2 BMDMs. Collectively with the evidence that M1 BMDMs had the highest capacity to acidify the medium, we concluded that MCT BMDMs presumably divert glucose-derived pyruvate to feed into the TCA and support mitochondrial metabolism. To our surprise, MCT BMDMs displayed the lower mitochondrial mass and potential when compared to all other BMDMs phenotypes (M0, M1 and M2a). Further studies will be needed, on one hand, to understand BMDMs mitochondrial biology and, on the other hand, to unravel if and how mitochondria orchestrate antitumour functions of MCT-stimulated macrophages.

In conclusion, our data reveal that MCT effectively induces iNOS-expressing antitumour macrophages that feature a mitochondrial metabolism, presumably fitted to the nutrient availability of the TME. We believe this thesis lays the groundwork for the design of therapeutic strategies that encompass functional and metabolic reprogramming of TAMs. Harnessing the myeloid compartment therapeutically could synergize with standard and emerging anticancer therapies to improve oncologic patient healthcare and survival.

Keywords: Tumour Microenvironment, Immune surveillance, Macrophages, Cancer Immunotherapy, Metabolism.

RESUMO

O microambiente tumoral é um ecossistema muito heterogéneo, constituído não apenas por células tumorais mas também por matriz extracelular, fibroblastos e ainda células do sistema imunitário, tanto da linhagem mieloide como da linfoide. Estas células do nosso sistema de defesa possuem a capacidade natural de vigiar e eliminar tumores em desenvolvimento, principalmente por meio das propriedades citotóxicas das células T. No entanto, as células imunológicas muitas vezes não conseguem controlar o crescimento desregulado dos tumores devido a mecanismos de evasão – por eles mesmos induzidos – que surgem e se desenvolvem ao longo da carcinogénese, prejudicando a imunidade antitumoral e comprometendo a sobrevivência do hospedeiro. Com efeito, à medida que o tumor se desenvolve, as próprias células malignas educam o estroma que as rodeia para superar os obstáculos da vigilância imunológica, tanto por meio de fatores solúveis imunossupressores, como por comunicações célula a célula inibidoras do sistema imunitário ou ainda através de competição metabólica por nutrientes. Estes factores, todos em conjunto, impedem a erradicação do cancro por meio do sistema imunitário.

Entre os elementos do microambiente tumoral que são directa ou indirectamente modulados pelas células malignas, destacam-se as células mieloides por geralmente constituírem a população imunitária mais representativa dos infiltrados imunes e, mais ainda, pela plasticidade funcional que demonstram, podendo exercer funções tanto antitumorais como protumorais, de acordo com os estímulos que recebem nos tecidos em que se encontram. Com isto em mente, verificámos que tem sido dada uma atenção desproporcional entre as células mieloides que promovem o crescimento de tumores (protumorais) em relação às células que os tentam combater (antitumorais). Por isso, estamos convencidos de que mais esforços devem ser feitos para perceber como é que podemos aumentar a atividade protetora das células mieloides no contexto do cancro. O conhecimento dos mecanismos utilizados pelo nosso sistema de defesas sempre foi fundamental ao longo da história para o desenho de terapias eficientes, em qualquer contexto clínico. Assim, o conhecimento biológico e consequente manipulação terapêutica das células mieloides podem colaborar com as terapias existentes contra o cancro, por exemplo a quimio- e a imunoterapia, para melhorar os cuidados de saúde e a sobrevivência dos pacientes oncológicos.

No sentido de estudar o potencial antitumoral das células mieloides, neste trabalho utilizámos um modelo ortotópico de células tumorais mamárias de ratinho (E0771) que nos permite estudar as actividades antitumorais das células mieloides *in vivo*. Tirando partido da capacidade inerente que estas células possuem de responder

a agentes de maturação, como ligandos dos receptores *Toll-like receptor* (TLR) e moléculas co-estimuladoras, pudemos demonstrar nesta tese que a injecção intratumoral de um ligando do TLR3 (Poly I:C) em combinação com um agonista da molécula co-estimuladora CD40 (tratamento MCT) foi capaz de induzir remissão total dos tumores implantados, na esmagadora maioria dos ratinhos tratados. Demonstrando posteriormente que este tratamento combinatório não é prejudicial, em si mesmo, para as células tumorais, concluímos que o efeito alcançado dever-se-ia ao impacto do tratamento no microambiente tumoral. Com o objectivo de dissecar os mecanismos subjacentes ao efeito marcante que alcançámos com este tratamento e tendo em consideração a capacidade inerente que as células mieloides têm para responder ao mesmo, eliminámos os macrófagos selectivamente e reparámos que o tratamento perdeu a sua eficácia. Concluímos, assim, que o efeito terapêutico era dependente da ação dos macrófagos. Analisando de seguida os infiltrados imunitários após o tratamento, observámos que os macrófagos associados ao tumor (TAMs) sobre expressaram duas moléculas pro-inflamatórias com propriedades antitumorais conhecidas, a saber TNFα e iNOS, por comparação com os ratinhos não tratados. Para estudar o papel que estas moléculas poderiam ter na resposta antitumoral mediada pelos macrófagos, utilizámos um sistema *in vitro* de macrófagos derivados da medula óssea de ratinhos. Após a diferenciação, tratámo-los com diferentes estímulos para podermos comparar o comportamento dos macrófagos estimulados com o MCT com macrófagos antitumorais (M1) e protumorais (M2a e M2c). Verificámos, em primeiro lugar, que os macrófagos MCT sobre expressaram igualmente TNFα e iNOS, dando credibilidade à nossa metodologia. De seguida, observámos que o sobrenadante (SN) dos macrófagos MCT era capaz de induzir morte das células E0771, por semelhança com o SN dos M1 e por diferença com o SN dos M2a e M2c. Para perceber se este efeito se devia à produção de TNFα, utilizámos o mesmo sistema em cima descrito com o suplemento de anticorpos monoclonais que bloqueavam a ação do TNFα. No entanto, a presença do anticorpo não foi capaz de aumentar viabilidade celular das células E0771. Para estudar a importância desta molécula *in vivo*, injectámos os mesmos anticorpos bloqueadores do TNFα concomitantemente com o tratamento MCT. Em sintonia com os resultados obtidos *in vitro,* não registámos qualquer diferença na eficácia do tratamento. Como abordagem alternativa, utilizámos clones das células E0771 resistentes à ação do TNFα. Após a transplantação ortotópica destas células e da injecção do MCT, reparámos que o tratamento foi igualmente eficaz. Estes resultados, no seu conjunto, levaram-nos a concluir que o TNFα não tem um papel importante enquanto mediador da resposta

antitumoral dos macrófagos. Procurámos, então, perceber se a expressão de iNOS poderia ser responsável pelas propriedades antitumorais dos macrófagos estimulados com o MCT. Aproveitando ratinhos geneticamente modificados para não expressar iNOS (ratinhos iNOS-ko), diferenciámos macrófagos *in vitro* e tratámo-los como em cima está descrito. Interessantemente, observámos que na ausência da expressão de iNOS as propriedades antitumorais dos SN dos macrófagos MCT estavam significativamente reduzidas, sugerindo que o MCT é capaz de induzir a expressão de iNOS em macrófagos para mediar a sua resposta antitumoral.

Impulsionados pela caracterização funcional dos macrófagos antitumorais MCT, e sabendo que o metabolismo celular pode ditar o resultado funcional das células imunes, investigámos os requisitos metabólicos dos macrófagos MCT. Para nossa surpresa, descobrimos que eles diferiam dos macrófagos M1, que eram marcadamente glicolíticos, e que, ao invés disso, exibiam uma dependência mitocondrial semelhante à dos macrófagos M2, perfil metabólico este que se pensa ser ajustado às características nutricionais do microambiente tumoral. Essa semelhança refletiu-se depois num conteúdo celular de ATP semelhante entre os macrófagos MCT e M2. Dadas estas semelhanças, perguntámo-nos quais seriam os substratos metabólicos que suportavam o metabolismo mitocondrial dos macrófagos MCT. Curiosamente, observámos que os macrófagos MCT eram capazes de captar glicose de forma semelhante aos M1 e que possuíam um conteúdo lipídico distinto dos M2. Juntando a evidência de que os macrófagos M1 tinham a maior capacidade de acidificar o meio de entre todos os macrófagos, concluímos que os macrófagos MCT não usam os lípidos como fonte de energia, como fazem os macrófagos M2, mas antes desviam o piruvato derivado da glicose para alimentar o TCA e o metabolismo mitocondrial. Para nossa surpresa, observámos que os macrófagos MCT apresentaram a menor massa mitocondrial e o menor potencial de membrana mitocondrial quando comparado com os outros fenótipos de macrófagos (M0, M1 e M2a). Futuros estudos mais detalhados permitir-nos-ão, por um lado, compreender a biologia das mitocôndrias dos macrófagos e, por outro lado, desvendar se e como é que as mitocôndrias orquestram as funções antitumorais de macrófagos MCT.

Em conclusão, o trabalho aqui desenvolvido demonstra que o tratamento MCT induz macrófagos antitumorais que expressam iNOS e que apresentam um metabolismo marcadamente dependente da mitocôndria. Acreditamos que estes estudos promissores estabelecem as bases para o desenvolvimento de estratégias terapêuticas que abranjam a reprogramação funcional e metabólica dos TAMs, com vista à sua combinação com outras terapias contra o cancro.

Palavras-chave: Microambiente Tumoral, Vigilância imunitária, Macrófagos, Imunoterapia contra o cancro, Metabolismo.

INTRODUCTION

1. The Tumour Microenvironment.

1.1 Cancer immunoediting.

Cancer is one of the major causes of death worldwide[1]. Rather than an isolated cell mass with aberrant genetic features, cancer tissues are constituted by a complex environment of distinct cell types – such as innate and adaptive immune cells, endothelial cells and stromal fibroblasts – integrated in a milieu of nutrients, cytokines and growth factors[2], altogether known as Tumour Microenvironment (TME)[3].

For many years, the immune compartment of the TME was considered to have a protective role in tumour development. Some pivotal studies from late 90s led to the cancer immunosurveillance hypothesis in mice, arguing a role of some molecules of the immune system, such as IFNγ and perforin, to control primary tumour development in immunocompetent hosts[4,5,6]. Later studies reported added layers of complexity in this surveillance mechanisms, suggesting an heterogenous process requiring immune effectors from both the innate and adaptive immune systems[7,8,9]. Several epidemiologic studies extended this hypothesis to humans[10–12] demonstrating that hosts with immune deficits have a higher probability of developing a variety of cancers, altogether supporting the critical role of the immune system to control nascent tumours. How then tumours occur in immunocompetent hosts? This puzzling question was untangled with experimental data holding the immune system not only is capable of destroying malignant lesions but also to sculp its immunogenicity[5,13,14]. By eliminating immunogenic tumour variants, immune cells are simultaneously selecting the less sensitive to an immune attack, thus facilitating the immune evasion of malignant cells[15].

Taken together, these studies constitute the experimental evidence of the "Cancer immunoediting" concept that holds the interactions between the immune system and transformed cells undergo three distinct processes: (a) *elimination*, that corresponds to immunosurveillance and elimination of malignant cells; (b) *equilibrium*, by which tumour cells with intrinsically high immunogenicity are eliminated whereas its counterparts with lower immunogenicity are left behind, surviving an antitumour immune response; and (c) *evasion,* during which tumour variants with acquired insensitivity to immunologic detection and elimination proliferate in an uncontrolled manner[16] (Fig. 1).

1.2 The Immune Compartment as Regulator of Disease Outcome

In addition to the experimental and epidemiologic studies described above, accumulating evidence shows that the presence of CD3ε+ T lymphocytes in the tumour

bed positively correlates with increased patient survival[17]. Some studies have refined this correlation to demonstrate that CD8+ T cells are the most relevant subset to better predict patient survival[18]. More recent studies appreciated the abundance of tumoricidal-like TAMs as a prognostic factor in breast cancer[19], strengthening the strong prognostic value of immune cell presence and antitumour activation at tumour sites. However, despite the protective functions the immune system can perform within the TME, tumour cells that evade the selective pressure of an immunologic attack effectively educate the surrounding stroma to promote tumour growth to malignancy. Through a variety of mechanisms, including immunomodulatory cytokine/chemokine production and membrane-bound molecules, malignant cells recruit and instruct immune cells to promote tumour progression, invasion of healthy surrounding tissue, angiogenesis and metastasis[20–24,25], while suppressing any immune cell-mediated cytotoxicity[26]. Collectively, these studies demonstrate immune cells are educated in cancer tissues with implications, either protective or detrimental, for tumour progression and patient survival[27]. Thus, the phenotype of the tumour-infiltrating immune cells can be determinant for oncologic disease outcome.

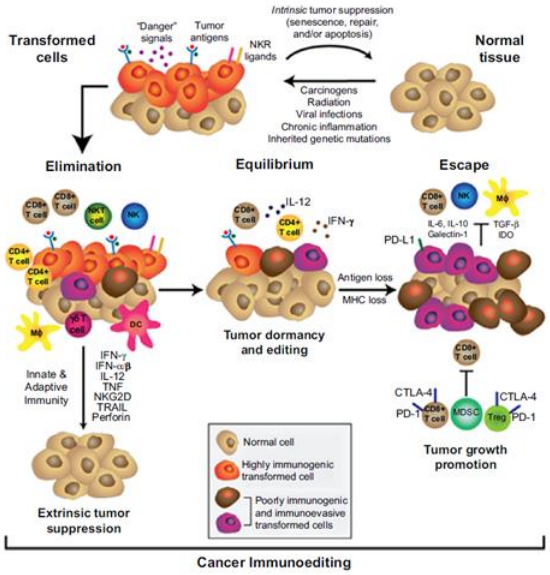

Fig. 1 – The cancer immunoediting concept. Cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immune cells work together to destroy developing tumours long before they become clinically apparent. If, however, a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its outgrowth is prevented by immunologic mechanisms. Editing of tumor immunogenicity occurs in the equilibrium phase. As a consequence of constant immune selection pressure placed on genetically unstable tumour cells held in equilibrium, cancer cell variants may emerge that become insensitive to immune effector mechanisms and/or induce an immunosuppressive state within the tumor microenvironment. These tumor cells may then enter the escape phase, in which their outgrowth is no longer blocked by immunity. These tumor cells emerge to cause clinically apparent disease (adapted from Schreiber et al.[16]).

Amongst the diversity of immune players found in the ecosystem of tumours, macrophages are one of the most represented populations[28,29] (Fig. 2, left) Many functional *in vitro* studies demonstrated that macrophages could perform tumour cell-killing activities, suggesting TAMs could display tumoricidal features in the tumour bed[30–32]. Nevertheless, a large body of evidence links the presence of macrophages in cancers with poor prognosis[33–35]. Indeed, TAMs generally support tumour progression through their interactions with tumour cells and stromal environment[36–39]. The functional diversity macrophages can display is a consequence of their remarkable capacity to sense a broad spectrum of environmental cues, including TLR ligands, cytokines and co-stimulatory molecules[40]. In the 90s, efforts to functionally characterize macrophages led to the binary classification of classical M1, that emerge in response to LPS and IFNγ and perform tumoricidal activities[41,42], and alternative M2 macrophages, programmed with IL-4 and IL-13 and capable to promote tumour growth[43,44]. This binary nomenclature, however, fails to effectively portray the phenotypic diversity of TAMs in the TME, driven by the multitude of polarizing cues present in malignant tissues[45,46] (Fig. 2, right). As such, a strong body of evidence indicates macrophages as plastic tumor infiltrating-immune cells that can respond accordingly to the signalling inputs they encounter in the TME[25]. This versatile feature of macrophages suggests selective macrophage manipulation might bring a new layer to the actual effective immunotherapeutic approaches to fight cancer.

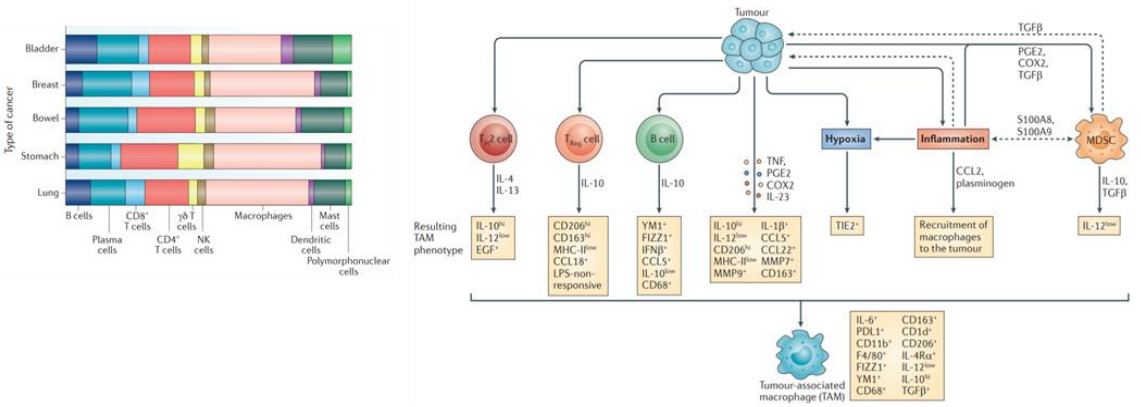

Fig. 2 – Macrophage infiltration and phenotype in the tumour microenvironment. Immune infiltrates vary according to cancer type. However, macrophages represent the major population infiltrating most human cancers (left; adapted from Cassetta and Pollard[47]). Tumour and stromal cells produce factors that drive the generation of multiple types of tumour associated macrophages (TAMs) (right; adapted from Gabrilovich et al.[25]).

1.3 Metabolic instruction of immunity in the TME.

In the past decade, ground-breaking reports unravelled the interplay between cellular metabolism and tumour immunity, adding another layer of complexity to

immune cell regulation in tumour tissues[48]. Cellular metabolism is an intracellular network of processes that regulate ATP production, protein and lipid synthesis and the balance of reducing equivalents (NAD(P)H and $FADH_2$)[49]. Central to this network is the mitochondria**,** which can oxidize glucose-derived pyruvate, fatty acids and amino acids, linking the TCA cycle with OXPHOS chain reactions to produce ATP[49] (Fig. 3, right). Mitochondrial metabolism is a highly efficient mode of ATP production used by cells whose primary requirements are energy and longevity. However, highly proliferative cells often upregulate aerobic glycolysis (also known as the Warburg effect[50]) to rapidly provide the cell the ATP and biosynthetic intermediates required for intensive proliferation and function[51].

Recent studies have underlined the importance of understanding the correlation between metabolism and immune functions, leading to the creation of the "immunometabolism" field. For instance, glucose metabolism is critical for immune cell activation and, particularly, antitumour phenotypes in dendritic cells (DCs), macrophages and T cells[52–54] and is considered as a hallmark metabolic feature in most immune cells, including classical M1 macrophages, undergoing rapid activation *in vitro* (Fig. 3, left). Within the TME, however, immune and cancer cells often share similar metabolic needs[55] and this may lead to local metabolic competition in which transformed cells usually outcompete their immune counterparts[51,56–59]. Accumulating data have demonstrated that tumour cells deprive the TME of glucose and amino acids, preventing immune cells to switch from OXPHOS to glycolysis and compromising their functions to control cancer growth[51,56]. Local metabolic competition can further promote the emergence of cancer facilitating immune subsets, such as myeloid-derived suppressor cells (MDSCs)[60] and regulatory T (T_{regs}) cells[61,62]. By contrast, other reports indicate that tumour-promoting immune cells, such as tolerogenic TAMs, MDSCs and T_{regs}, feature an activated mitochondrial metabolism usually dependent on fatty acid oxidation (FAO)[63–66], similarly to the metabolic programmes displayed by alternative M2 macrophages *in vitro* (Fig. 3, left).

Altogether, these reports suggest the metabolic requirements of immune cells in the TME greatly influence their effector functions to control tumour growth. In the cancer setting, increased mitochondrial metabolism appears as a metabolic adaptation to the TME that circumvents nutrient deprivation and sustains pro-tumour immune functions, while starving antitumour immune effectors struggle to meet the cellular needs they require to fight cancer. Thus, enhancing immune cell fitness in cancer tissues by metabolic modulation could be a powerful weapon to develop more effective combinatorial treatments for the anticancer defence.

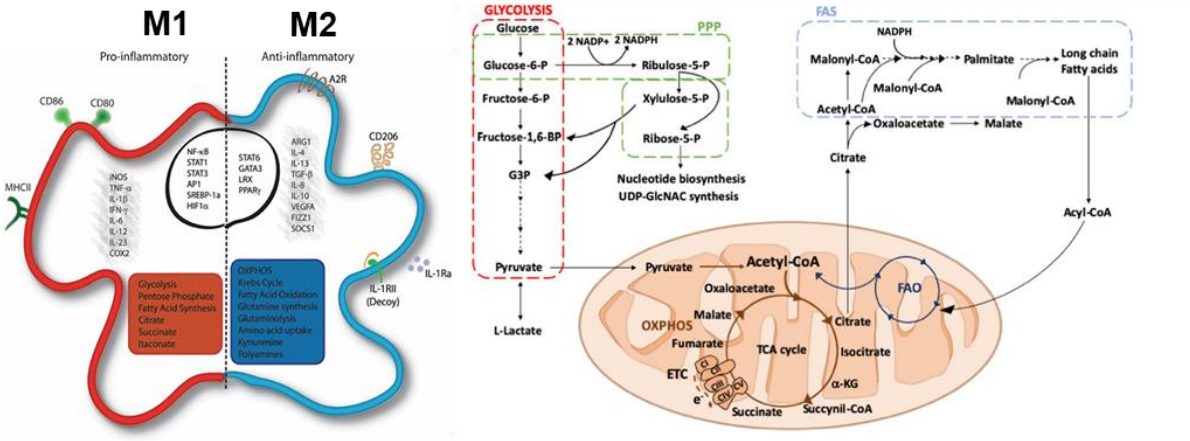

Fig. 3 – Molecular and metabolic signatures of macrophage activation. Pro-inflammatory stimuli induce the activation of specific functional pathways and a metabolic reprogramming toward glycolysis, the pentose-phosphate pathway, and fatty acid synthesis (M1-like macrophages). Anti-inflammatory macrophages have a distinct functional phenotype and display enhanced OXPHOS metabolism and fatty acid oxidation (adapted from Viola et al.[67]).

2. Tumour Immunotherapy.

2.1 The breakthrough of immunotherapies.

The standard-of-care anticancer therapies include surgery, chemo- and radiotherapy[68] which can induce tumour shrinkage and eradication and, most importantly, ameliorate the overall survival and lifestyle of cancer patients. However, such therapeutic interventions can also inadvertently provide a potent selective pressure for the clonal expansion of resistant variants with improved fitness and malignant potential[69]. As a consequence of therapeutic failure, tumours relapse and deteriorate cancer patient health and survival, highlighting the urgency for new anticancer modalities that potentiate the elimination of clonally distinct malignant cells.

As a glimpse of hope, the past decades witnessed the advent of another anticancer therapeutic approach – immunotherapy – provided by convincing reports in the immuno-oncology research field. By targeted activation of immune cells that infiltrate cancer tissues, interferons and IL-2 demonstrated clinical benefits for oncologic patients and got FDA approvals later on[70]. This was followed by clinical trials demonstrating the efficacy of checkpoint-blockade immunotherapies (described below), that unleash the antitumour potential of T cells by releasing them from inhibitory receptor signalling[71–73]. These experiments summarize the feasibility and efficacy of lymphocyte-targeting anticancer therapies, either by direct activation or release from inhibition, and proved the power of harnessing the latent potential of a patient's immune system in cancer therapy.

Cancer cell spatial and temporal heterogeneity, reflected in aberrant genomic profiles, cell behaviour and metabolism, is one of the major drivers of malignant progression and cancer-related deaths[74,75]. The successful efforts to mobilize the host's immune system to recognize and eliminate the cancer cells have changed the target focus from the highly flexible tumour cell to a relatively more rigid player, such as the immune system. Thus, key advances in the immune-oncology research field shifted the paradigm of cancer therapy as we knew it. The major breakthroughs in immunotherapy are explored below.

2.2 T cell-based immunotherapies prove the principle.

Cancer cell elimination is mostly operated by CD8 T cells[5] which are capable of eliminating developing tumours. Upon recognition of tumour antigens, T cells can mount a robust immune response to recognize and kill malignant cells[76]. T cells require antigen presentation by MHC molecules, on antigen presenting cells (APCs), to the corresponding TCR, on naïve T cells, and also costimulatory stimulation through CD28 and B7 molecules for full activation[77,78]. T cell activation is tightly regulated by inhibitory receptors, such as CTLA-4 and PD-1 (also known as immune checkpoints), to avoid collateral damage and autoimmunity[79]. Both molecules halt T cell activation upon engagement with their respective ligands (B7-1/2 for CTLA-4 and PD-L1/2 for PD-1). In the context of cancer, the engagement of these receptors with their ligands hinders antitumour immunity by negatively regulating tumour-reactive CD8 T cell cytotoxic activity. The pioneer work of Profs. James P. Allison and Tasuko Honjo, respectively on CTLA4 and PD-1, led to development of monoclonal antibodies to block these inhibitory receptors – Immune Checkpoint Blockers (ICB). Due to the striking results and the outstanding benefits ICB has demonstrated on human health care, having saved thousands of lives, Profs. Allison and Honjo were awarded the Nobel Prize of Physiology and Medicine in 2018. Other approaches to take advantage of the unique capacity of T cells to recognize and kill tumour cells are the adoptive cell therapy (ACT)[80] and CAR T cells[81]. One ACT modality requires the isolation of tumour-infiltrating lymphocytes (TILs) from a patient, expansion of those cells and then re-injection into the circulation of the same patient. This modality has been able to induce durable complete regressions in patients with melanoma[82,83], a highly immunogenic type of cancer. Melanoma tumours usually present a high mutational load that can generate tumour antigens recognised by T cells. Thus, to expand this modality to other cancer types with lower mutational load, T cells started to be genetically engineered. The most successful modification on T cells gave origin to the CAR-T cell therapy, which involves genetic manipulation of a patient's T cells to specifically target and

respond to a given antigen. CAR T cell therapy provides a unique way to specifically instruct T cells towards tumour surface antigens, leading to impressive clinical results in CD19-expressing lymphomas[84] and promising results in other malignancies[85,86].

The striking results and the major benefits T cell-based immunotherapies have demonstrated on the clinical setting confirmed immunotherapy as a valuable weapon in the war against cancer. Regrettably, only few patients can benefit from the currently available T cell-centred immunotherapies[87–90]. Given the evolving nature of immune/cancer cell interactions in the TME, a plethora of immune evasion mechanisms arises to impede T cell antitumour immunity and facilitate cancer progression[91]. These include neoplastic-cell intrinsic and extrinsic factors, the latter related to other components of the TME such as T_{regs}[92] and immunosuppressive myeloid cells[93–95]. Macrophages, for instance, can upregulate PD-L1 to directly hamper CD8 T cell antitumour activity[36,96]. Together, immune evasion mechanisms to immunotherapeutic treatments that seek to restore or enhance T cell antitumour defence call for novel TME-targeting immunotherapies in order to achieve deeper and durable responses.

2.3 Macrophage-targeting immunotherapies come of age.

Macrophages are innate immune cells critical to maintain tissue homeostasis[97]. Upon an insult, macrophages can rapidly evoke an immunological response that links innate and adaptive immune cells[98,99] to eliminate the danger and restore homeostatic conditions. In the context of cancer, however, malignant cells hijack macrophage properties in benefit of their own prosperity[100]. Macrophage role in cancer has been strongly associated with the promotion of multiple aspects of tumorigenesis at the primary and metastatic sites. From cancer initiation to malignant progression and dissemination, macrophages can stimulate angiogenesis, invasion of the healthy surrounding tissue and intravasation, simultaneously suppressing local and systemic antitumour immunity[101–107]. Macrophage tumour facilitating activities are moreover reflected in their capacity to promote resistance to standard of care[108,109,110] and T cell-focused anticancer therapies[111,112]. Together with reports indicating high density of cells with macrophage-associated markers in the tumour bed is associated with poor clinical outcome[35,113], these studies point macrophage-impacting therapies as appealing alternatives for cancer treatment. Such therapies can be roughly divided into those that ablate TAMs survival and recruitment and those that reprogramme their pro-tumoural activities towards tumoricidal-like phenotypes (Fig. 4), altogether favouring antitumour immunity and therapeutic response.

Critical to the survival of macrophages and recruitment to neoplastic tissues are the molecules CSF-1[114,115] and CCL2[21,116,117]. Different strategies targeting both signalling axis with monoclonal antibodies (mAbs) have proven effective to control tumour outgrowth[86,120] and synergized with standard therapies[119,121] and immunotherapies[122,123]. Despite the consistency of pre-clinical and clinical data, a variety of roadblocks ultimately limit the efficacy of these strategies, including compensatory resistance pathways that correlate with decreased response and durability of the blockade[124,125], concomitant depletion of TAMs and tissue-resident macrophage populations important to maintain homeostasis[126] and tumour rebound after cessation of therapy[127].

To overcome these hurdles, reprogramming tumour macrophages towards an antitumour phenotype, instead of depleting protumour TAMs, became an attractive approach to augment the efficacy of macrophage-targeting anticancer therapies. Despite being generally pro-tumoral, TAMs can also be active immune cells in the antitumour defence[97,128] which suggests that macrophage phenotypic plasticity could be explored to restore their antitumour properties[129]. It further points the pitfall of macrophage-depleting strategies, as both antitumour and protumour macrophage subsets would be depleted. Instead, macrophage reprogramming is a targeted strategy that provides a unique opportunity to shift the tumour immune infiltrates from a tolerogenic state to one that fights cancer cells in synergy with T cell-enhancing drugs, such as checkpoint inhibitors. A key advantage of reprogramming is that it avoids the drawbacks of toxicity and long-term systemic elimination of macrophages. Different adjuvant strategies have been explored and tested in pre-clinical and clinical models. Toll-like receptor stimulation, which has fundamental roles in the activation of the innate immune system[130], with synthetic bacterial particles or viral nucleic acids has been tested in cancer models in order to reprogram TAMs towards tumoricidal macrophage phenotypes[131,132]. For instance, TLR7 agonist intra-tumoural delivery has shown antitumour activity in several models and correlated with histological tumour regression and increased myeloid and lymphoid infiltration[133–135]. TLR3 ligands have also been described to skew tumour-protective macrophages towards tumour-fighting counterparts, restricting tumour growth in mouse models of cancer[136]. In addition, macrophage stimulation towards antitumour states can also be achieved with anti-CD40 agonistic stimulation. CD40 is a receptor that belongs to the TNF receptor superfamily and it is expressed on myeloid and B cells[137]. Upon engagement with its ligand CD40L, mainly expressed on T cells[138], CD40 downstream signalling increases the expression of MHC molecules and the production of pro-inflammatory mediators, such as IL-12, involved in T helper and cytotoxic cell priming.

Interestingly, different types of macrophage activation induce not only functional but also metabolic reprogramming, typically from an OXPHOS to a glycolysis-centred metabolism, which is important for activation and immunogenic functions of macrophages[139,140]. Understanding the functional and metabolic regulation of tumour-infiltrating macrophages mediated by different adjuvant modalities will be important for the development of novel macrophage-based anticancer approaches[141] to foster the antitumour potential of TAMs and enhance the synergy with other immunotherapeutic treatments.

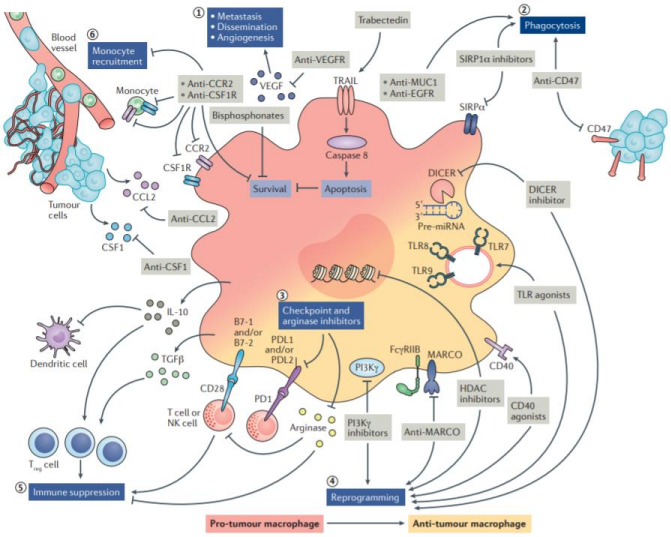

Fig. 4 – Targeting and reprogramming TAM protumoural activities. Protumoural tumour-associated macrophage (TAM) activities that have been targeted in either preclinical models or therapeutic trials in humans (adapted from Cassetta and Pollard et al.[47]).

OBJECTIVES

Macrophages are critical mediators of oncologic disease outcome due to their dynamic interplay with cancer, stromal and immune cells. Owing to the tumour growth-promoting and T cell-immunosuppressive functions they perform in the TME, strategies to deplete in macrophages have been embraced to enhance antitumour immunity. Nevertheless, and building on the inherent phenotypic flexibility macrophages display, we believe there has been a disproportioned attention to protumour compared to antitumour macrophages, that can be effectively induced with the appropriate stimuli and become strong allies in the battlefield of cancer tissues. Deeper knowledge on how we can manipulate macrophages to instruct an immune-permissive and cancer cell-hostile TME may inspire combinatorial treatments to potentiate both arms of the immune system to jointly eradicate malignant lesions.

Here, we first aimed at manipulating macrophages with TLR ligands and agonistic stimulation of co-stimulatory receptors to unleash an antitumour response. Upon accomplishing this objective, we went on to dissect the molecular determinants expressed by activated macrophages that directly impaired tumour cell proliferation.

In addition, we proposed to study the metabolic requirements of antitumour macrophages in order to find new avenues to exploit therapeutically. Since macrophage metabolic demands tightly correlate with effector functions, we aimed at unravelling the metabolic programs coordinating macrophages antitumour defence.

METHODS

1. Mice and Tumour Cell Lines.

C57Bl/6J (B6) female WT mice were purchased from Charles River Laboratories. *Nos2*−/− mice were kindly provided by Margarida Correia-Neves (Life and Health Sciences Research Institute (ICVS), Braga, Portugal). Mice were maintained in specific pathogen-free facilities at IMM. Animals were 6–18 weeks of age and aged-matched within 3 weeks. No randomization or blinding was performed when mice were allocated into experimental groups.

The E0771 murine breast cancer cell line was purchased from Tebu-Bio. Cells were tested for mycoplasma contamination regularly (Mycoalert Mycoplasma Detection kit from VWR, following manufacturer's instructions) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) FCS (Gibco; Thermo Fisher Scientific) and 1% (vol/vol) penicillin/streptomycin (P/S; Sigma/Merck) – complete DMEM (cDMEM) hereafter – at 37ºC and 5% CO_2. For passages, E0771 cells are detached using TrypLE Express (Gibco; Thermo Fisher Scientific) with 1ml for a small 25ml flask, and 2ml for a medium 75ml flask, during 5min at 37ºC in the incubator. For Long-term storage of E0771 cell lines, they were resuspended in FBS with 10% DMSO and kept at -80ºC until further use (1x10^6 cells/freezing vial). After thawing, we performed a washing step with cDMEM to remove DMSO and tumour cell lines were maintained in cultured as stated above. When defrosted before tumour implantation, E0771 cells were passed once and expanded immediately.

To isolate the E0771 TNFα-resistant clones, E0771 cells were cultured with 300ng/ml of TNFα (Peprotech) for three weeks. Fresh TNFα was replenished every other day. A limiting dilution was performed to isolate single cell clones from the parental E0771 cells proliferating in the presence of TNFα. The 3 most proliferating clones were expanded and further subjected to a Cell Viability Assay (see below) to confirm acquired and maintenance of TNFα resistance.

2. Tumour implantation *in vivo*, Monitorization and Treatment.

Female WT mice received a single sub-cutaneous injection of $1x10^6$ of either E0771 or E0771 TNFα-resistant tumour cells on the 4th mammary fat pad on the left side. Tumours were monitored daily with a calliper from the timepoint they were palpable. Tumour volume was estimated as (length x width x width)/2 (mm^3). When implanted tumours reached 50-100mm^3 of volume, mice were treated every three days with intra-tumoural (IT) injections of a 50μl solution made of 50μg of the TLR3 agonist poly I:C (InvivoGene; tlrl-picw) and 15μg of an agonistic anti-CD40 mAb (Bio X cell;

clone FGK4.5/FGK45), hereafter termed myeloid cell-treatment (MCT). Mice that did not develop visible tumours were excluded from the analysis. Mice were killed by CO_2 narcosis at desired timepoints or when reached the maximum tumour volume permitted (established by the institution and national ethic guidelines).

3. Generation of bone marrow-derived macrophages (BMDMs).

Mice were sacrificed by CO_2 narcosis and disinfected with 70% ethanol. A midline abdominal incision and vertical incisions on both legs were made to separate the skin from underlying tissues. Femurs were separated from the tibia and the pelvis, cleansed with 70% ethanol and further processed in sterile conditions. To isolate BM haematopoietic progenitors, femurs were flushed on both sides with 10ml syringes and 0,5mm needles filled with RPMI 1640 supplemented with 10% FCS and 0,2% P/S (BM-medium hereafter) to a 50ml falcon tube. The resulting solution of BM-progenitors was filtered through a 100μm nylon cell strainer (Falcon/Corning) to a new 50 ml falcon tube to exclude debris. BM-progenitors were then diluted in Trypan Blue (Sigma) and quantified with a Neubauer chamber. $4x10^6$ BM-progenitors were seeded in petri dishes with 10ml of BM-medium supplemented with 50ng/ml of macrophage-colony stimulating factor (M-CSF; Peprotech) for 3 days, at 37ºC with 5% CO_2. On day 3, 10ml of BM-medium + 50ng/ml of M-CSF/petri dish was added for further 3 days. On day 6 of differentiation, we distributed $2x10^6$ macrophages/well in a 6-well plate, in 2ml of BM-medium. On day 7, BMDMs were polarized for 6 or 24h, at 37ºC with 5% CO_2, with the following conditions: M0 – unstimulated; M1 **–** LPS (50ng/ml) + IFNγ (50ng/ml); M2a – IL-4 (50ng/ml) + IL-13 (50ng/ml); M2c – IL-10 (50ng/ml) + TGFβ (2ng/ml); MCT – Poly I:C (10μg/ml) + anti-CD40 (5μg/ml). BMDM phenotypes were confirmed by flow cytometry analysis (see “Cell preparation and flow cytometry analysis” section below). All cytokines were purchased from Peprotech. LPS was purchased from InvivoGene.

For long-term storage of BM-progenitors after isolation, they were resuspended in FBS with 10% DMSO and kept at -80ºC (5x10^6 cells/freezing vial). After thawing, we performed a washing step with BM-Medium to remove DMSO and BM-progenitors were counted and seeded in petri dishes, as mentioned above.

4. Cell preparation and flow cytometry analysis.

E0771 tumours were resected, mechanically cut into pieces with scissor in Eppendorf tubes and afterwards incubated with 2ml of DMEM or RPMI containing only Collagenase I (0.4mg/ml) and IV (1mg/ml) (both from Worthington Biochemical) and DNase I (10ug/ml) (Sigma/Merk) for 30min, 37ºC, under agitation (1200rpm). Two ml of RPMI supplemented with 1x Sodium Pyruvate (ThermoFisher), 1x Non Essential Amino Acids (Alfagene), 1x Hepes (Alfagene), 1x β-Mercaptoethanol (Alfagene), 10%

FCS and 0,2% P/S – complete RPMI (cRPMI) hereafter – were added to stop the action of collagenases and DNase I. Cell suspensions were further dilacerated on a 100μm nylon cell strainer, cleansed and filtered to 5ml FACS tubes. Cells were centrifugated (1500rpm/5min/ room temperature (RT)) and resuspended in 500ul of RBC Lysis Buffer (Biolegend) was added for 2min at RT to osmotically lyse erythrocytes. Cells were washed in cRPMI, counted and distributed at maximum $5x10^6$ cells/well in 96-well plate V-bottom. Cell were FcR blocked with anti-mouse CD16/CD32 (clone 93; eBioscience/Thermo Fisher Scientific) at 1/200 in cRPMI for 10min, RT. For extracellular surface staining, the fluorescent antibodies mix is prepared in cRPMI and added on the top of each cell suspension for 30min-1h at RT in the dark. For detection of dead cells, we incubated cells 50ul/well Zombie Aqua (ThermoFisher) 1/400 in PBS, for 20min at RT, protected from light. For intracellular cytokine and iNOS staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer set (eBioscience/ThermoFisher Scientific), following the manufacturer's instructions, and then incubated with fluorescent antibodies for 30 minutes at RT, protected from light. See supplementary table 1 for the list of antibodies used.

To confirm BMDMs *in vitro* polarization and profile their metabolic requirements, we distributed $1x10^5$ cells/well in 96-well plate V-bottom. Extracellular and Intracellular stainings were performed as described above. We used fluorescent dyes for metabolic profiling as it follows: Bodipy for lipid content (ThermoFisher; 9.5μM final concentration, prepared in PBS and incubated at RT), 2-NBDG for glucose uptake (146μM final concentration, prepared in PBS and incubated at RT), the cell permeable MitoTracker Green dye (ThermoFisher; 1μM final concentration, prepared in BM-medium and incubated at 37ºC) was used to selectively stain all undamaged mitochondria, regardless of the membrane potential, and the cell permeable TMRE (ThermoFisher; 1μM final concentration, prepared in BM-medium and incubated at 37ºC) was used to trace active mitochondria. All samples were acquired on a FACS Fortessa (BD Biosciences) and data were analysed with FlowJo software V10.6.0.

5. Cell viability assay.

$1x10^4$ E0771 cells in cDMEM were seeded on a 96-well plate flat bottom for 2/3h to allow adherence. For recombinant cytokines, 1:4 increasing concentrations from 0.3ng/ml to 300ng/ml were added to a final volume of 200μl/well. For MCT, 1:5 TLR3L increasing concentrations from 0,016μg/ml to 50μg/ml with fixed 2.5μg/ml of anti-CD40 were added to a final volume of 200μl/well. In the assays with, we replaced the medium after tumour cell adherence with 100μl of each BMDM-subtype SN.

Nutrient supplementation of SN with 10% of FCS (Gibco; ThermoFisher Scientific) and/or 25mM glucose (Sigma) was performed directly on the wells. Monoclonal antibodies anti-TNFα (InVivoMab, XT3.11, Cat#: BE0058) and anti-IFNγ (InVivoMab, XMG1.2, Cat#: BE0055) were added on the top of the SN. After 24h, regardless of the assay, the medium was removed and replaced with 80µl of Cell Titer Blue (VWR) diluted 1:20 in cDMEM for 1h at 37ºC, 5% CO_2. Cell viability reading was performed in Microplate Reader Tecan Infinite M200, with the following parameters: 10s orbital shaking, 590nm excitation and 560nm emission of fluorescence.

6. Detection of tumour cell apoptosis and/or death.

$5x10^4$ E0771 cells in cDMEM were seeded on a 24-well plate overnight in 300µl of cDMEM. On the following day, the medium was removed and replaced with BM-macrophage SN for 48h at 37ºC. Cells were afterwards detached with TrypLE Express 5min at 37ºC and transferred to 5ml FACS tubes. Annexin V (Invitrogen) staining was performed for 1h at RT and shaking, protected from light. 7-AAD (Biolegend) staining was performed for 15min at RT, protected from light. Samples were analysed by flow cytometry as described above. Detection of tumour cell apoptosis and/or death: Annexin V positive staining indicates that cells have entered in an apoptotic process. A positive 7-AAD staining indicates the plasma membrane is compromised and it is an accurate indicator of cell death.

7. DNA cell cycle analysis.

$5x10^4$ E0771 cells in cDMEM were seeded on a 24-well plate in serum-free medium to reset the cell cycle, overnight at 37ºC. On the following day, serum-free medium was replaced with the experimental conditions (BMDM supernatant or MCT in cDMEM) for 48h at 37ºC. After incubation, cells were fixed with ethanol 70% overnight and shaking. Cells were then incubated with PI (Merck Life sciences) and RNase (Sigma) for 1h at RT. Samples were analysed by flow cytometry as described above. This approach allows to quantify DNA content/cell cycle phase/cell and to detect cell cycle arrest by changes in PI staining. The higher peak corresponds to the G1 phase, the second peak to G2/M and the DNA content in between considered S-phase. Any staining below G1 phase indicates DNA fragmentation and, thus, cell death.

8. Scenith Protocol.

After day 8 post-differentiation and polarization, we distributed 1.10^5 BMDMs/well in 96-well plates. Macrophages were treated during 30 minutes with the following conditions: Control (BM-medium), 2-Deoxy-D-Glucose (DG, 100mM), Oligomycin (Oligo, 1µM) or the combination 2-DG + Oligo at the final concentrations before mentioned. For this last condition 2DG is added for 15min and Oligo

supplemented for the next 15min. Puromycin (10µg/ml) is added during the last 15 minutes of the metabolic inhibitors treatment. After puromycin treatment, cells were washed in cold PBS and stained with a combination of mouse Fc receptors blockade and fluorescent antibodies against different surface markers (see supplementary table 1) during 30 minutes at 4ºC in PBS 1X 5% FCS, 2mM EDTA (FACS wash buffer). After washing with FACS wash buffer, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer (eBioscience/ThermoFisher Scientific) following manufacturer instructions. Intracellular staining of puromycin using an Alexa Fluor 647 labelled anti-puromycin mAb diluted in Perm wash was performed at 4ºC for 1h. Metabolic inhibitors, puromycin and the antibody anti-puromycin were all kindly provided by Dr Rafael Arguello (CIML, Marseille, France). Samples were acquired on a FACS Fortessa (BD Biosciences) and data were analysed with FlowJo software V10.6.0.

To characterize the different energetic requirements of macrophages, we quantify the impact on protein synthesis (puromycin MFI) of inhibiting a given metabolic pathway compared to complete inhibition of all ATP (supplementary figure 5). The percentual of glucose dependence (Gluc. Dep.) quantifies how much the translation levels are dependent on glucose oxidation. Gluc. Dep. is calculated as the difference between puromycin MFI levels in 2-DG treated cells compared to control (Co), divided by the difference in puromycin MFI upon complete inhibition of ATP synthesis (2-DG + Oligo) compared to control cells. In a similar fashion, percentual mitochondrial dependence (Mitoc. Dep) quantifies how much translation is dependent on mitochondrial oxidative phosphorylation. Mitoc. Dep. is defined as the difference in puromycin MFI levels in Oligomycin treated cells compared to control relative to PS levels upon full inhibition of ATP synthesis inhibition (treatment Z) also compared to control cells. Finally, glycolytic capacity is defined as the maximum capacity to sustain puromycin MFI when mitochondrial OXPHOS is inhibited. The corresponding formulas below were adapted from Arguello et. al[142]:

Co= *GeoMFI of anti-Puromycin-Fluorochrome upon Control treatment*

DG= *GeoMFI of anti-Puromycin-Fluorochrome upon 2-Deoxy-D-Glucose treatment*

O= *GeoMFI of anti-Puromycin-Fluorochrome upon Oligomycin A treatment*

Z= *GeoMFI of anti-Puromycin-Fluorochrome upon DG+O treatment*

$$Glucose\ Dependence\ (Gluc.\ Dep.) = \frac{100\ (Co - DG)}{(Co - Z)}$$

$$Mitochondrial\ dependence\ (Mitoc.\ Dep.) = \frac{100\ (Co - O)}{(Co - Z)}$$

$$Glycolytic\ capacity\ (Glyco.\ Cap.) = 100 - \left(\frac{100\ (Co - O)}{(Co - Z)}\right)$$

Total level of translation (puromycin MFI) in the control condition correlates with the global metabolic activity of the cells. Using Scenith protocol, we can calculate “dependency” parameters, that underline essential cellular pathways that cannot be compensated, and the metabolic “capacity” of macrophages, that indicate the maximum compensatory capacity of cells to exploit alternative pathways when a particular one is inhibited.

9. ATP content.

To quantify ATP content of BMDMs after 24h of polarization, cells were plated on a 6MW plate and stored at -20ºC after polarization treatment. Next, cells were thawed for 30 minutes on ice and lysed with 50ul/well of extraction buffer (EB; 6M guanidine-HCl (Sigma-Aldrich, G3272), 100mM Tris/HCl (Sigma-Aldrich, B9755, pH 7.8, 4mM EDTA (Sigma-Aldrich, ED2SS) and transferred to 1,5ml Eppendorf tubes. Cell lysates were incubated on ice for 5 minutes and then snap-frozen and lastly incubated at 95ºC for 3 minutes and subsequently centrifuged at max speed for 10 minutes at 4ºC. Supernatant was collected and protein quantification was performed using the BioRad Protein Assay Kit, according to the manufacturer’s instructions. To measure ATP content, we used luminescent luciferase-based ATP Determination Kit (Molecular Probes/ThermoFisher Scientific, catalogue nº A22066) according to the manufacturer’s instructions. ATP content was determined by establishing an ATP concentration standard curve.

10. pH assessment

pH quantification was performed with pH strips (Sigma). One drop/square of each solution was applied on the strip and the yielded colour code was compared with a standard scale provided by the manufacturer. A high precision pH meter (SI analytics, ref. ba77143e01) was used to validate pH strips readout.

11. Statistical analysis.

Data analysis was done with GraphPad Prim version 8. Applied analyses are indicated in the corresponding legends. No statistical methods were used to predetermine sample size. Differences with $P<0.05$ were considered statistically significant. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.001.

RESULTS

1. Intra-tumoural delivery of Myeloid Cell-Treatment induces tumour regression in a macrophage-dependent manner.

Given that macrophages are highly versatile constitute the most representative compartment of the tumour immune infiltrates[29,107], we asked if we could programme them with antitumor properties. To do so, we transplanted $1x10^6$ E0771 cells (TNBC cell line) in the mammary fat pad of WT mice. When tumour volume reached between 50-100mm^3, mice were treated with an intra-tumour administration of a TLR3L (Poly I:C) in combination with an agonistic anti-CD40 (MC-Treatment, hereafter termed MCT) every three days (Fig. 1a). This regimen effectively induced tumour eradication in most treated mice (Fig. 1b, left panel), dramatically augmenting overall survival when compared to the not-treated (NT) group of mice (Fig. 1b, centre panel). Importantly, we detected a fast MCT-induced tumour growth inhibition already 3 days after treatment, as depicted in the T3/T0 tumour size ratio (Fig. 1b, right panel). suggesting an efficient anti-tumoural immune response early after treatment.

TLR agonistic stimulation can have detrimental effects directly on tumour cells[143,144]. To investigate this in our model, we incubated E0771 cells *in vitro* with a TLR3 ligand alone, anti-CD40 alone or both in combination. E0771 cell viability was not significantly affected compared to the control with cDMEM (Supplementary Fig. 1a). We further confirmed that MCT does not induces E0771 cell death in itself (Supplementary Fig. 1b), excluding any direct cytotoxic activity of MCT on tumour cells and further suggesting that MCT induces cancer cell eradication by modulating the tumour myeloid ecosystem.

Macrophages can be redirected with antitumour activities by activation of pro-inflammatory signalling pathways such as TLRs and co-stimulatory molecules like CD40[9,145]. To determine whether macrophages were necessary for MCT therapeutic efficacy, we injected MCT-treated mice with clodronate-encapsulated liposomes intravenously (i.v.) and intraperitoneally (i.p.) to deplete both systemic and tumour-associated macrophages, respectively (Fig. 1c). Interestingly, we significantly delayed tumour cell eradication upon macrophage depletion in MCT-treated mice (Fig. 1d, left panel) and we lost the tumour growth inhibition 3 days after MCT (Fig. 1d, right panel), pointing macrophages as key mediators of the MCT-induced tumour elimination.

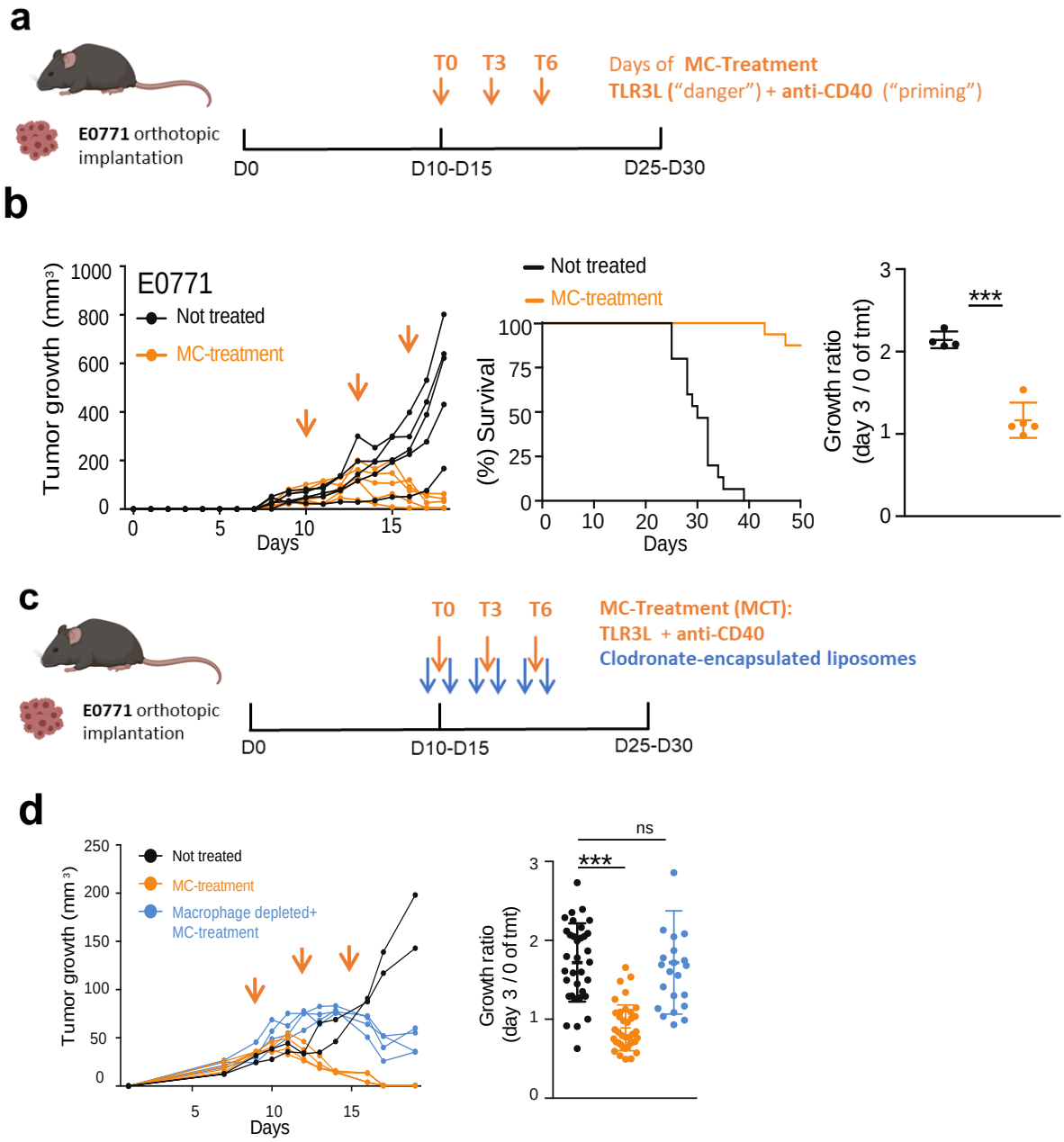

Figure 1 – Intra-tumoural delivery of Myeloid cell-Treatment Induces Tumour Regression in a Macrophage-Dependent Manner. a, Schematic showing E0771 implantation on the mammary fat-pad of syngeneic WT mice (day 0) and subsequent treatment schedule with 50ug Poly I:C + 5ug anit-CD40 (MC-Treatment, MCT). T0, T3 and T6 are the days of treatment. **b**, Longitudinal tumour growth curves of individual mice (n=5 mice per group). Mice with implanted E0771 tumours were injected every 3 days. Arrows indicate intratumoural delivery of MCT (left). Kaplan-Meier curve showing the survival of tumour-bearing mice not-treated or MCT-treated. Data from several independent experiments (centre). Tumour size ratio T3/T0 was calculated diving the tumour volume after day 3 of MCT treatment by the tumour volume at the baseline before treatment (day 0; n=5 mice per group; right panel). **c,** Schematic showing the treatment schedule of syngeneic E0771 tumour-bearing mice with MCT treatments plus macrophage depletion using clodronate-encapsulated liposomes. **d**, Longitudinal tumour growth curves of individual mice. Mice with implanted E0771 tumours were injected every 3 days. Arrows indicate intratumoural delivery of MCT. Data shown is representative of several independent experiments (left panel). Tumour size ratio T3/T0 was calculated as in **b,** right panel. Significance was calculated with two-tailed Mann-Whitney *U*-test. Tmt – Treatment.

2. Myeloid Cell-Treatment induces macrophage activation towards an antitumour phenotype.

The aforementioned complete abrogation of the T3/T0 tumour size ratio in the absence of macrophages led us to hypothesise that macrophages acquire antitumour activities early after MCT. To validate our assumption, we harvested tumours from MCT and NT mice 3 days after treatment and analysed the phenotype of myeloid cells by flow cytometry. Macrophages were herein identified as CD45+ CD3- CD19- NK1.1- Ly6G- CD11b+ Ly6c+/- (Supplementary Fig. 2). When compared to its counterparts, MCT-activated macrophages displayed elevated expression of TNF-α and iNOS (Fig. 2a,b, top panel), which are known to have direct or indirect antitumoural functions in the TME[146–148]. We further noticed an increased presence of iNOS+ macrophages within cancer tissues (Fig. 2b, bottom panel). These data indicated that MCT activates macrophages towards an TNF-α+ iNOS+ antitumoural-like phenotype and gave us plausible mechanistic insights of macrophage antitumour activities.

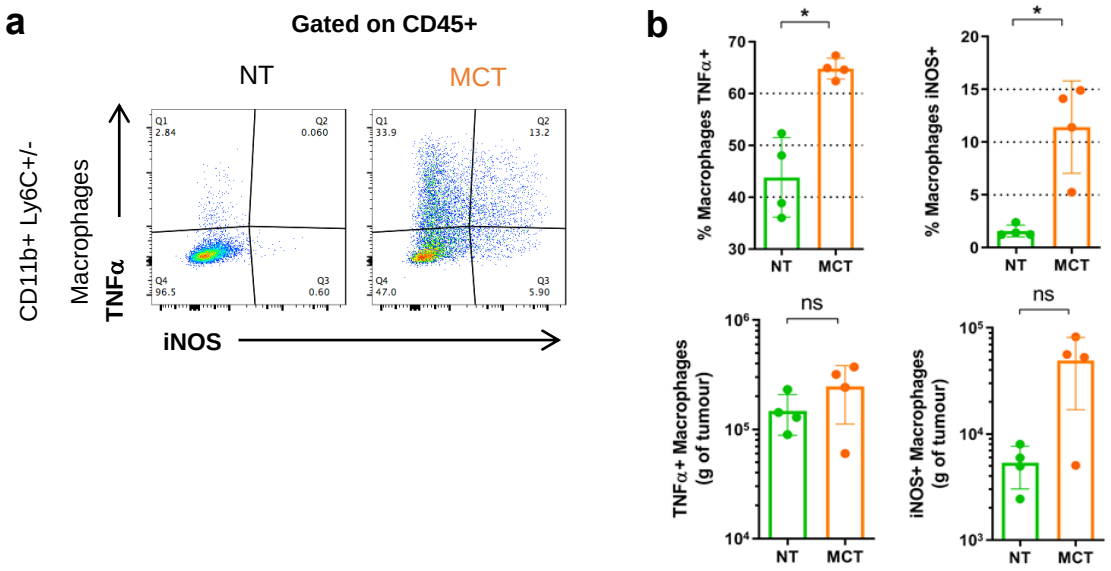

Fig. 2 – Myeloid Cell-Treatment induces macrophage activation towards an antitumour phenotype. a, Flow cytometry of harvested tumour-infiltrating macrophages from not-treated or MCT-treated mice, 3 days after the first MCT injection. Macrophages were identified as CD45+ CD3- CD19- NK1.1- CD11b+ Ly6C+/-. **b,** Percentage (top) and number of cells/g of tissue (bottom) of TNFa+ and iNOS+ macrophages from NT and MCT tumours. Data is representative of several independent experiments. Significance was calculated with two-tailed Mann-Whitney *U*-test.

3. MCT-polarized macrophages induce tumour cell killing *in vitro*.

To dissect the antitumoural activities of MCT-polarized macrophages, we used *in vitro* bone marrow-derived macrophages (BMDMs) After differentiation, BMDMs were polarized with different stimuli to induce distinct activation states: M1, M2a, M2c

and MCT macrophages (Fig. 3a. See methods section “isolations of BMDMs” for more details). This panel of macrophage phenotypes enabled us to functionally compare MCT macrophages with the dichotomy pro-inflammatory (tumoricidal) M1 vs anti-inflammatory (pro-tumoural) M2 macrophages, as introduced above. Following polarization, we evaluated their phenotype by flow cytometry. TNFα and iNOS, hallmarks of pro-inflammatory macrophages, were substantially increased in M1 compared to M0 (unstimulated), M2a and M2c (Supplementary Fig. 3). This is consistent with the available literature[149], confirming an effective polarization. MCT macrophages presented a similar antitumoural phenotype as M1, although with moderated expression of both TNFα and iNOS. Importantly, these data show *in vitro* MCT macrophage phenotype resembled the one of *in vivo* MCT-activated macrophages, as shown in Fig. 2a, demonstrating BMDMs as a reliable tool to dissect antitumoural activities induced by MCT. Moreover, the phenotypic similarities between *in vitro* MCT and M1 further suggest a tumoricidal functional overlap.

Therefore, we reasoned that MCT BMDMs produce cytotoxic soluble factors detrimental for tumour cells. To validate this hypothesis, we cultured E0771 cells in the presence of the supernatant (SN) of MCT BMDMs and quantified cell viability as a readout of cytotoxicity. When compared to the control M0 BMDM-SN, we noticed a dramatic decrease of E0771 viability in the presence of MCT macrophage-SN (Fig. 3b, top panel). To rule out the possibility of tumour cell starvation due exhausted SN resulting from the consumption of the nutrients by activated BMDM during the 48h of polarisation, we incubated E0771 cells with MCT BMDMs-SN either alone or with nutrient supplementation. The later included glucose and FCS equally concentrated as in normal culture medium. Nevertheless, tumour cell viability decrease remained unchanged (Fig. 3b, bottom panel). Altogether, these data indicate that MCT BMDMs produce soluble factors that impair tumour cell viability.

To further dissect the mechanism limiting tumour cell viability in the presence of MCT BMDM-SN, we questioned if this was due to prevention of tumour cell proliferation. We did a cell cycle analysis based on the propidium iodide (PI) staining of double-stranded DNA, followed by flow cytometry analysis (see methods for more details). After incubation of E0771 cells with MCT BMDM-SN, we detected an increased staining of fragmented DNA, which is an indicator of cell death, without signs of cell cycle arrest (Fig. 3c, left panel). While M1 SN induced higher cell death, the other phenotypes (M0, M2a, M2c) had no impact on E0771 cell cycle. This led us to culture E0771 cells with SN of various BMDM phenotypes and analyse tumour cells with an Annexin V and 7-AAD staining, by flow cytometry, to assess the percentage of apoptotic and necrotic cells, respectively. When compared to the control, we detected

increased tumour cell apoptosis and necrosis in the presence of MCT BMDM-SN to similar level than those induced by M1 BMDM-SN (Fig. 3d). As expected, SN from the other phenotypes (M0, M2a, M2c) had no impact on E0771 survival. Thus, these data revealed that MCT BMDM-derived soluble factors induce tumour cell death.

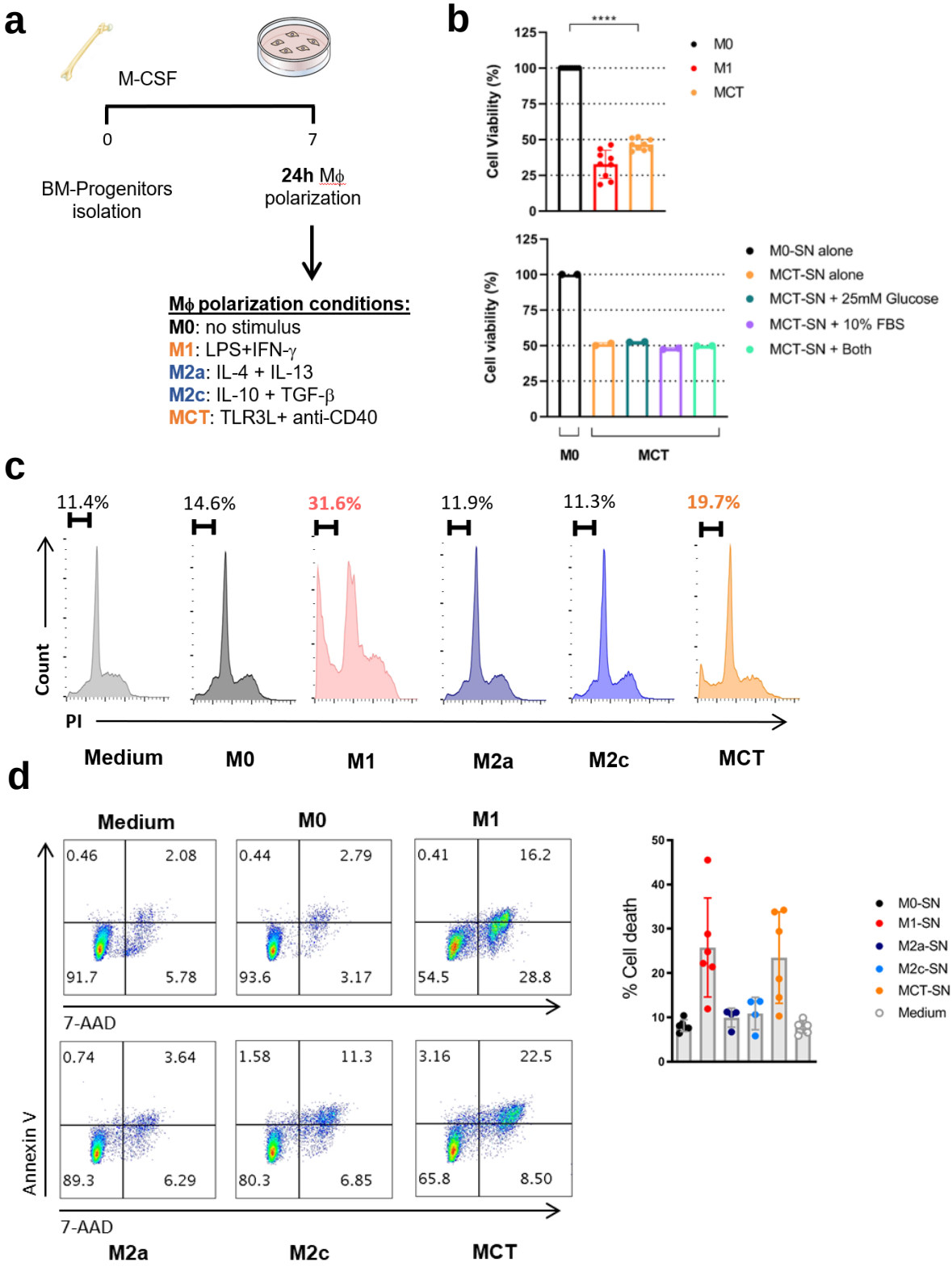

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Fig. 3 – MCT-polarized macrophages actively induce tumour cell killing *in vitro*. a, Schematic representation of *in vitro* differentiated BMDMs. After 7 days of culture in the presence of 50ng/ml of M-CSF, BMDMs are polarized with different conditioned media for 24h. MCT BMDMs are polarized with the same treatment injected *in vivo*. **b**, Tumour cell viability in the presence of BMDM-SN (top) with or without nutrient supplementation (bottom). **c**, Flow cytometry of tumour cells cultured with BMDM-SN for 48h. Tumour cells were analysed with a PI staining. Percentages shown indicate the sub-G1 phase and correspond to the staining of fragmented DNA, a readout of cell death (left). **d**, Flow cytometry of tumour cells cultured with BMDM-SN for 48h. Tumour cells were analysed with Annexin V and 7-AAD stainings to detect apoptotic and necrotic cells, respectively (left). Cell death was quantified as the percentage of 7-AAD+ tumour cells (right). Significance was calculated with two-tailed Mann-Whitney *U*-test.

4. MCT macrophage antitumoural function is mediated by iNOS.

Having demonstrated that MCT effectively unleashes the antitumour potential of BMDMs through secretion of cytotoxic soluble factors, we aimed at finding the soluble mediator(s) of such phenotype. The *in vitro* and *in* vivo data both suggested TNFα as a candidate (Fig. 2,b; Supplementary Fig. 3), a cytokine with described antitumour activities[150,151]. To assess whether E0771 cell line was sensitive to TNFα, we cultured E0771 cells in the presence of increased concentration of recombinant TNFα and we observed a dose-dependent decrease of tumour cell viability (Fig. 4a), giving rational for TNFα being the cytotoxic soluble molecule produced by MCT BMDMs. We next cultured E0771 with MCT BMDM-SN with or without blocking anti-TNFα monoclonal antibody. To our surprise, blocking anti-TNFα signalling failed to increase tumour cell viability in the presence of either M1 or MCT BMDM SNs (Fig. 4b). To assess the potential role of TNFα *in vivo*, we administered the blocking anti-TNFα antibodies on MCT mice but neither T3/T0 tumour size ratio nor overall tumour growth rate were affected (Fig. 4c). Finally, as an alternative *in vivo* approach, we selected a TNFα-resistant E0771 cell line *in vitro* (Supplementary Fig. 4; see methods for more details) as an alternative *in vivo* approach, This TNFα-resistant E0771 cell line was implanted into WT mice that subsequently received MCT. Consistent with the *in vitro* data obtained with the blocking anti-TNFα antibody, the TNFα-resistant E0771 tumours were eradicated with the same kinetic as the E0771 tumours upon MCT (Fig. 4d). Altogether, these results exclude TNFα as the cytotoxic molecule mediating antitumor activities of MCT-activated macrophages and made us look for another mediator of MCT-macrophage tumour-destructive activities.

Given the elevated expression of iNOS in MCT-activated macrophages *in vitro* and *in vivo* (Fig. 2,b; Supplementary Fig.3), we questioned whether this enzyme could mediate MCT macrophages antitumour activity. iNOS is an enzyme encoded by the *Nos2* gene that produces the gaseous free radical nitric oxide (NO), capable of

inducing tumour cell death[152]. To define its functional role in our model, we generated *in vitro* macrophages from iNOS knock-out (iNOS-ko) mouse bone marrow. Then, we cultured tumour cells with iNOS-ko or WT MCT BMDM-SN. We found iNOS expression is partially responsible for the capacity of M1 or MCT BMDM-SN to kill E0771 tumour cells. In the absence of iNOS, BMDM-SNs were impaired in their ability to affect tumour cell viability. This suggested that iNOS might be a key mediator of MCT-polarized macrophage antitumour activity.

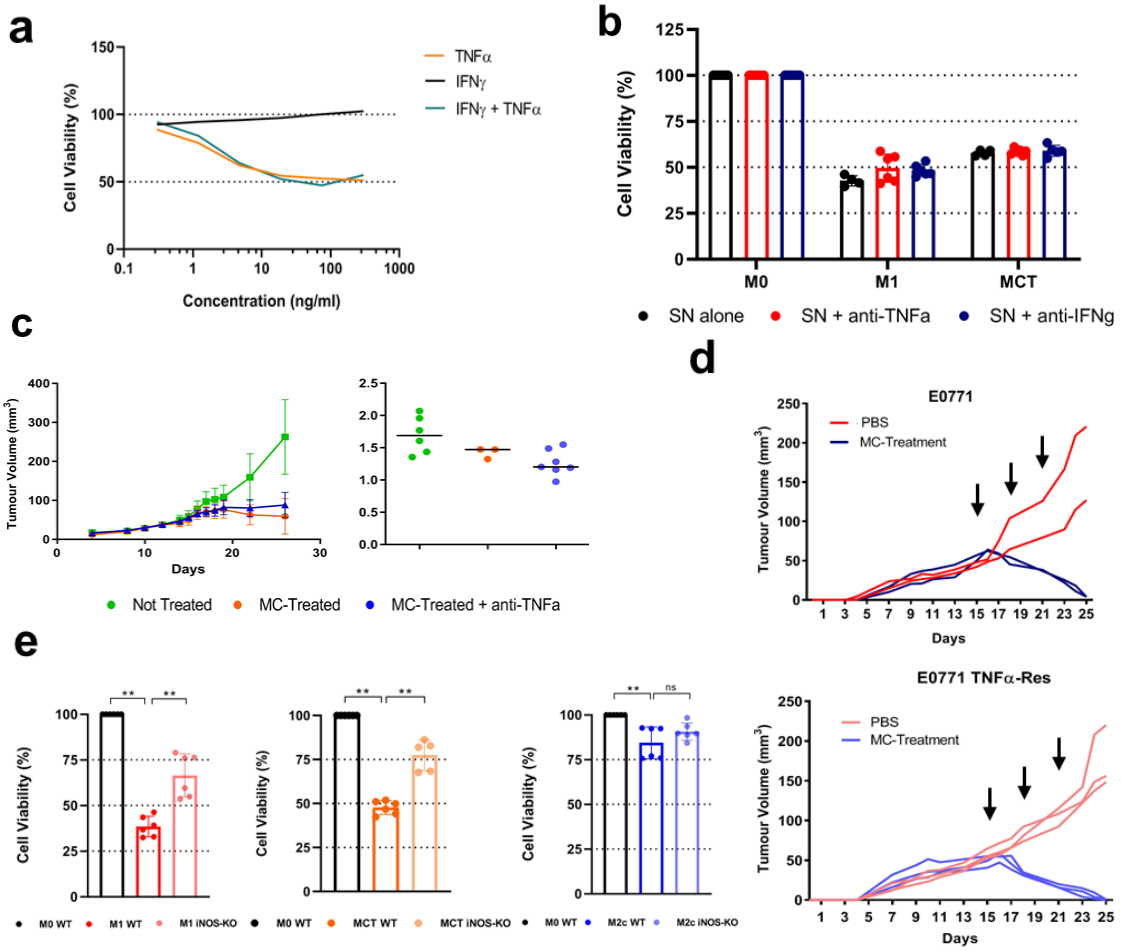

Fig. 4 – MCT macrophage antitumoural function is mediated by iNOS expression. a, E0771 cell viability cultured with recombinant TNFα, IFNγ or both cytokines in combination. Cell viability is calculated relative to the control (tumour cells + cDMEM). **b,** E0771 cell viability after incubation with BMDM-SN, alone or the presence of blocking anti-TNFα or IFNγ monoclonal antibodies. Cell viability is calculated relative to the control for each experimental conditions. Data from two independent experiments. **c,** Syngeneic WT mice with E0771 tumours within the range of 50-100 mm3 were injected with MCT alone or in combination with anti-TNFα blocking antibodies (n= 6, 3 and 7 for not treated, MCT-treated and MCT-treated + anti-TNFα antibodies, respectively; left panel). Tumour size ratio T3/T0 was calculated diving the tumour size after day 3 of MCT treatment by the tumour volume at the baseline before treatment (right panel). **d,** Syngeneic WT mice were implanted with either E0771 (n=2 mice per group) or E0771 TNFα-resistant (n=3 mice per group) cell lines and MCT-injected when tumours reached between 50-100 mm3. Arrows indicate the days of MCT-injections, administered every 3 days. **e,** E0771 cell viability after 48h of culture with BMDM-SN of WT or iNOS-ko mice. Data from two independent experiments. Significance was calculated with two-tailed Mann-Whitney *U*-test.

5. Antitumour MCT macrophages display an oxidative metabolism.

In response to environmental cues, such as TLR agonists and cytokines, macrophages undergo profound metabolic adaptations in ways that correlate with and sustains their immunologic activities[48]. With this in mind, we went on to study the metabolic requirements of MCT macrophages in order to unravel new avenues we could explore to enhance antitumour functions. Pro-inflammatory (tumoricidal) M1 BMDMs display a glycolytic metabolism whereas anti-inflammatory (protumoural-like) M2 rely on a mitochondrial oxidative metabolism (OXPHOS) as source of energy and metabolic intermediates[139,153]. Therefore, having demonstrated a tumoricidal functional overlap between MCT and M1 BMDMs, we investigated whether the similarities extended to their metabolic profiles.

To characterize macrophage energetic requirements, we used the Scenith protocol, a newly developed flow cytometry method to profile energetic metabolism (EM) with single cell resolution[142]. Briefly, Scenith protocol takes advantage of puromycin incorporation in nascent peptides as readout of protein synthesis level. Once protein synthesis level and ATP content are kinetically coupled[142], detection of puromycin incorporation in nascent protein by fluorescence acts as a surrogate of cellular energetic status. The principle of Scenith is then to incubate a given sample in parallel with specific inhibitors of known EM pathways (Supplementary Fig. 5a). If a cell population is energetically dependent on a particular pathway, its ATP content will immediately drop and so will its protein synthesis level. Then, using puromycin fluorescence levels (puromycin MFI), we can profile the distinct energetic requirements across macrophage subsets (Supplementary fig. 5b; and see methods for more details).

Upon inhibition of glucose metabolism by 2-DG the capacity of all the phenotypes of BMDMs to sustain protein synthesis was compromised demonstrating that BMDMs rely on glucose-dependent metabolism (Fig. 5a, left panel). This was further reflected on the close to 100% glucose dependency (Fig. 5a, right panel). Then, we found that inhibition of mitochondrial respiration by oligomycin differentially affected M1 and M2 BMDM. While M1 BMDMs presented protein synthesis levels higher than the control (without inhibitor), M2 BMDMs had lower levels than the control (Fig. 5a, left panel). Thus, M1 macrophages appeared independent of mitochondrial metabolism and displayed the highest glycolytic capacity among all subsets, whereas M2 BMDM presented a significant percentage of both glycolytic capacity and mitochondrial dependency (Fig. 5a, right panel). These results recapitulated previous reports on *in vitro* macrophage metabolic profiling that point M1 as glycolytic and M2 as OXPHOS-dependent[139,153], thus validating Scenith as a reliable tool to study macrophage

metabolism. Surprisingly, however, MCT BMDMs displayed a similar metabolic profile to M2 rather than M1 BMDMs, with similar glycolytic capacity and mitochondrial dependency (Fig. 5a, right panel). In turn, MCT BMDM ATP content was similar to M2 BMDMs and both were significantly higher than M1 BMDMs, consistent with the higher capacity of mitochondrial metabolism to produce energy compared to glycolysis[154]. Collectively, these data suggest antitumour MCT macrophages display mitochondrial OXPHOS activity, resembling protumour (M2-like) rather than antitumour (M1-like) macrophage metabolic profile.

These unexpected findings prompted us to dissect the mitochondrial requirements of MCT BMDMs. To do so, we used metabolic dyes that allow the characterization of cell metabolic features by flow cytometry (see methods for more details). First, we sought to understand the contribution of fatty acid oxidation (FAO) for MCT mitochondrial metabolism since lipid breakdown has been associated to both antitumoural[145] and protumoural[40] macrophages. To do so, we used the Bodipy dye that quantifies lipid droplets, which are ubiquitous and dynamic organelles that store neutral lipids (such as triglycerides and cholesterol esters). MCT BMDMs showed a similar content of lipid droplets as M1 BMDM and both were 2-fold higher than M0 and markedly increased compared to M2a (Fig. 5c, top left panel). This is consistent with the idea that cells that breakdown lipids for proliferation and function have lower lipid droplet content compared to those which do not oxidize lipids to satisfy their metabolic needs[64]. Thus, we excluded FAO as the main mitochondrial fuel in MCT BMDMs and concluded MCT and M2 BMDMs rely on different intermediates for mitochondrial metabolism. Given that MCT BMDMs have a considerable glycolytic capacity, we hypothesized that MCT macrophages would preferentially breakdown glucose to pyruvate, that would convert to acetyl-CoA to feed the TCA. To test this, we measured glucose uptake using a fluorescent non-metabolized glucose analogue 2-NBDG. Indeed, we noticed a 2-fold increase of glucose uptake in MCT compared to M2 BMBMs, although M1 BMDMs presented the highest (Fig. 5c, top right panel), consistent with the very high glycolytic capacity they display (Fig. 5a, right panel). Furthermore, the low pH level (pH 6) of M1 BMDM-SN (Fig. 5d) suggest they adopt the Warburg effect and divert pyruvate to produce lactate while MCT BMDMs, which SN was pH 7.5 (Fig. 5d), may use pyruvate as a precursor of acetyl-CoA to feed into the mitochondrial OXPHOS. Altogether, these data point MCT BMDMs use mitochondria similarly to M2 macrophages, although differing in the substrates they supply to the TCA. These intriguing findings led us to hypothesize that MCT and M2 macrophages would have similar mitochondrial features, such as mitochondrial mass and/or membrane potential, and distinct to those of M1. To investigate this, we first used the

MitoTracker Green dye as a readout of mitochondrial mass. Surprisingly, MCT macrophages had the lower mitochondrial mass when compared to M0 and M2, the later presenting the higher amount, and rather presented a similar phenotype to M1 (Fig. 5c, bottom left panel). We then reasoned that MCT would have a higher mitochondrial membrane potential compared to M1 given their similar mitochondrial mass but their different mitochondrial dependency. To measure mitochondrial membrane potential, we used the TMRE dye that specifically quantifies the membrane potential of mitochondria. Strikingly, MCT macrophages presented the lower membrane potential when compared to all other macrophage phenotypes while M1 counter-intuitively displayed a similar membrane potential to M2 macrophages. More studies are necessary to dissect the mitochondrial features of MCT BMDMs. Alternative approaches, as discussed below, will further give us a more comprehensive knowledge about MCT cellular metabolism. Altogether, these findings add a metabolic layer to the complexity of MCT-activated macrophage phenotype.

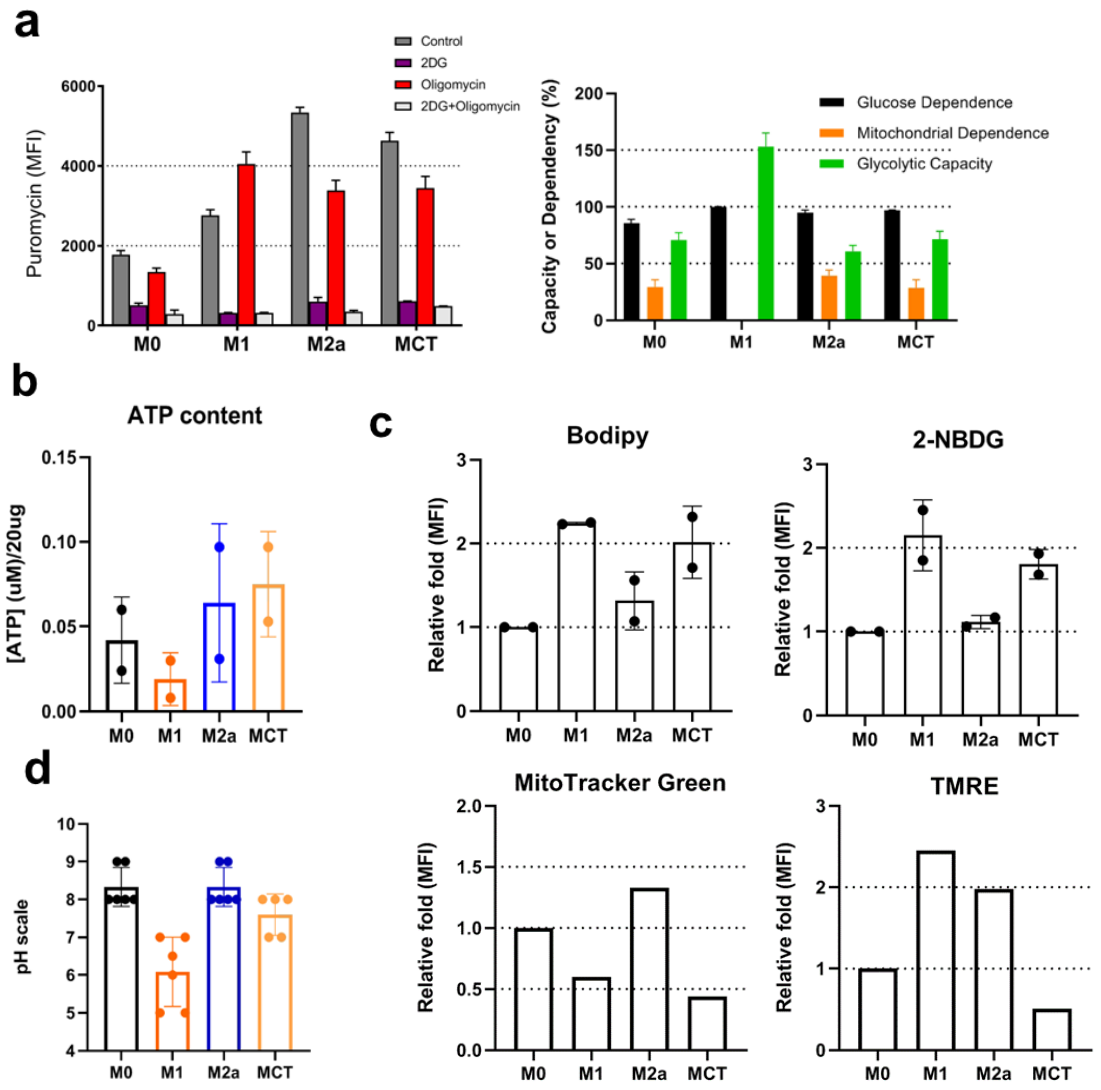

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Fig. 5 – Antitumour MCT macrophages display an oxidative metabolism. a, Metabolic profile of BMDMs with Scenith protocol. Puromycin staining is a readout of translation and, thus, ATP production (left). The metabolic pathway capacity/dependency of each macrophage subset is calculated with puromycin MFI (right; see methods for more details on the calculations). Data representative of three independent experiments. **b**, ATP content of BMDMs. The amount of ATP is relative to the amount of total protein of each BMDM subset. Data from two independent experiments. **c**, Neutral lipid content (Bodipy), glucose uptake (2-NBDG), mitochondrial mass (MitoTracker Green) and mitochondrial membrane potential (TMRE) of BMDMs after 24h of polarization were acquired with flow cytometry analysis. Data from two independent experiments for Bodipy and 2-NBDG. **d**, pH quantification of BMDM-SN after 24h of polarization with pH strips. Data from several independent experiments.

DISCUSSION

Macrophage therapeutic potential

The presence and phenotype of immune cells in the TME is a valuable predictor of the clinical outcome of oncologic patients[17,19]. Within the immune compartment of TME, T lymphocytes are professional killers of neoantigen-expressing tumour cells[156]. As the tumour evolves, interactions between cancer cells and the surrounding stroma can annihilate T cell antitumour function and thus facilitate tumour progression. A better understanding of the mechanisms underpinning such immunosuppressive tumour ecosystem, that encompass complex cellular networks and metabolic constraints, can bring new inroads for the development of more effective cancer immunotherapies. Among the stromal components of cancer tissues that can modulate the nature of T cell responses and ultimately impact tumour growth, macrophages have attracted a lot of attention since they constitute the largest immune population infiltrating the majority of solid cancers[29]. Functional plasticity is a hallmark of TAMs and their phenotype is determined in a cancer type-dependent manner to dictate either pro or antitumour functions[157]. Similarly to other immune subsets, like γδ T cells[158,159], an immune cell type that is a long-lasting interest in Silva-Santos lab, macrophage contribution for cancer outcome is not restricted to its direct impact on tumour cells but also extends to the communication with other immune subsets, since they often orchestrate the bridge between innate and adaptive responses[99,160]. Therefore, to compromise macrophage ability to launch a multi-layered antitumour response that would threaten cancer progression, malignant cells deploy several mechanisms to educate TAMs with features that, on one hand, promote tumour growth, invasion of surrounding healthy tissue and systemic dissemination while, on the other hand, block effective T cell activation and neoplastic cell killing. To know that macrophages are “rapid learners”, versatile immune cells that polarize accordingly to the surrounding environmental cues[149] makes them a very appealing target for cancer immunotherapy.

The potential to modulate macrophage response with TLR or co-stimulatory molecules has been studied in cancer mouse models and these molecules have been proposed has adjuvants for cancer immunotherapy[131,161]. In this work, we show the feasibility and efficacy of the MCT combinatorial treatment (TLR3 ligand + agonistic anti-CD40 antibody) to endow macrophages with antitumour functions to ultimately drive tumour eradication in a mouse model of TNBC. Importantly, we demonstrate MCT-activated macrophages are critical mediators of tumour growth delay *in vivo* and display tumoricidal activities *in vitro.* MCT was capable of triggering long-term tumour eradication in most treated mice through the induction of a strong and long-lasting anti-

tumour T cell response. Thus, given that most research and clinical data link have put light to the pro-tumour activities of TAM, we decided to take advantage of this unique mouse model to study anti-tumour TAMs as they act *in vivo* and to dissect the tumoricidal molecular determinants responsible for their antitumour effector functions.

Antitumour MCT macrophages molecular determinants

Upon treatment, *in vivo* TAMs produced TNFα and iNOS, which are inflammatory molecules with controversial activities in the TME[148,162]. TNFα is known to participate in tumour cell killing as the binding of TNFα to its receptor can activate a pro-apoptotic downstream pathway and induce tumour cell death[163]. In fact, we have shown that TNFα impairs the viability of the E0771 cell line *in vitro*. TNFα may also act in the education of the TME, as it is a key cytokine capable of blocking protumoural gene expression in TAMs[147] and also enhance CD8 T cell antitumour responses[164]. By contrast other models have pointed TNFα as a critical tumour-promoting cytokine[165], altogether highlighting the complexity of TNFα signalling activities in the TME. In this report, we undertook to elucidate the role of TNFα in the antitumour functionality of MCT-activated TAMs in the E0771 TNBC mouse model. To our surprise, despite the expression of TNFα in M1 and, to a lesser extent, in MCT BMDM, anti-TNFα blocking mAb failed to protect tumour cell viability when cultured with the SN of either M1 or MCT BMDM. Moreover, the results obtained *in vivo* with blocking anti-TNFα mAbs or with a TNFα-resistant E0771 tumour cell line are consistent with the *in vitro* studies, suggesting a neglectable role for TNFα upon MCT, in this model. The blockade of cytokines with mAbs has two major drawbacks: first, the effectiveness of mAbs *in vivo* is difficult to ascertain. Second, blocking mAbs inactivate all cytokine available regardless of the cellular origin. Considering that several immune populations can produce TNFα after activation[166], and indeed produce in our model (data not shown), with distinct and non-redundant functions of TNFα derived from myeloid and lymphoid cells in some contexts[167], it could be difficult to quantify the contribution of TNFα derived specifically from MCT-activated macrophages. Nevertheless, the 3 days window of macrophage tumour-limiting activities in our model could suit blocking mAbs methodology, as it did with others[168]. To overcome the hurdle of the unknown efficacy of *in vivo* mAbs, additional experiments with TNFα knock-out mice will consolidate our results about the molecular basis of MCT-activated macrophage antitumour functions. This approach could be complemented with lineage-specific TNFα ablation by crossing TNFflox/flox mice with LysM-Cre or CD4-Cre mice to obtain TNFα deficiency in the myeloid or lymphoid compartment, respectively. However, given that changes

macrophages operate in a discrete time period early after treatment to impair tumour growth, the plain KO mice can strengthen and clarify MCT-stimulated macrophage antitumour properties. Noteworthy, a recent study identified the loss of both TNFα and IFNγ signalling on tumour cells as major immune evasion mechanisms to CD8 T cell cytotoxic killing[169]. Interestingly, MCT can induce the elimination of a TNFα and IFNγ–resistant tumour cell line indicating that the robust and multi-layered antitumour response unleashed by MCT is capable of eliminating aggressive tumour variants that escape TNFα- and IFNγ-mediated immune surveillance. These results led us to look for another macrophage-dependent determinant of tumour regression.

The expression of iNOS, an enzyme encoded by the *Nos2* gene, is responsible for the production of NO. This gaseous free radical can have contrasting and diverse signalling functions in cancer tissues, modulating tumour cell fitness directly and also immune responses. It was reported that iNOS can promote tumour cell apoptosis, typically through caspase activation, chromatin condensation or DNA fragmentation[170,171], and mediate immune cell cytotoxicity[172,173]. iNOS signalling on tumour cells can also contribute for genetic instability and thus increase the potential of neoantigen presentation, that would favour antigen-specific T cell responses[174,175]. However, elevated iNOS expression in a cohort of TNBC patients was also associated with poor survival and increased tumour aggressiveness[176]. Furthermore, NO-mediated signalling can shape the immune myeloid landscape of malignant tissues. iNOS expression in myeloid cells has also been implicated in promoting T cell responses[177,178] and its deficiency in myeloid cells can diminish the efficacy of ACT[177]. In contrast, *Nos2* expression in TAMs is associated with its suppressive capacity[178–180] and iNOS inhibition can restore CD8 T cell function in the presence of macrophages[180]. The data presented here favour a protective role for iNOS expression in macrophages that support their tumoricidal activities *in vitro*, after MCT-polarization. In addition, recent single-cell RNA sequence analysis from our lab indicated *Nos2* expression on MCT-activated macrophages *in vivo* as the 2nd most differentially expressed gene, when compared to not treated counterparts (data not shown). Experiments with iNOS knock-out mice will enable us to assess the importance of iNOS expression in TAMs at various stages of the tumour progression after MCT. Interestingly, tolerogenic myeloid cells often upregulate arginase1 (ARG1), an enzyme that degrades the amino acid L-arginine and halt antitumour defence[181,182]. L-arginine usage is shared with iNOS that instead catabolizes L-arginine to L-citrulline, yielding NO and orchestrating macrophage metabolic rewiring towards an inflammatory phenotype[183]. In fact, NO can alter the TCA cycle and reroute pyruvate away from mitochondrial metabolism,

underlining the critical crosstalk between metabolic programming and functional polarization. From a therapeutic standpoint, inhibiting ARG1 could increase the availability of L-arginine in the TME and enhance MCT-activated macrophages antitumour response. The tight relationship between cellular metabolism and immune effector functions led us to dissect the metabolic requirements of MCT-polarized macrophages.

Metabolism as a guiding force for immunity

It is well-established that immune cells undergo metabolic reprogramming during activation, which is critically linked to their need to proliferate and perform effector functions. However, this adaptation is frequently hindered due to competition for substrates, particularly in the TME. Activated immune cells, including macrophages, and proliferating tumour cells share glucose dependency to thrive and the latter often outcompetes the first, leading to immune cell dysfunction and impaired antitumour immunity. Also, immune cells have to cope with an unfavourable TME enriched in tumour cell metabolic waste, such as lactate, that have been shown to affect myeloid and lymphoid cells responses[184,185]. Moreover, protumoral and immunosuppressive immune subsets in the TME have been reported to use mitochondrial oxidative metabolism as the source of energy and intermediates required for normal cellular activities. It is plausible that this protects protumoral immune players from metabolic competition with cancer cells and allows them to adapt and thrive in a hostile environment such as the tumour ecosystem. For example, Specifically, Angelini et al. observed that under high lactate levels, Tregs down-regulate glycolysis and increase mitochondrial respiration, resulting in augmented NAD production. This metabolite then contributes to the conversion of lactate into pyruvate that can be latter used for biosynthesis and energy production, revealing the ability of Tregs to resist in a lactate-rich TME and to preserve their suppressive ability[186]. Thus, elucidating the metabolic programming of anti-tumour TAM and dissecting the cues that can control these phenomena may highlight novel immunoregulatory events that govern the nature of the immune response. In the present study, we sought to understand the metabolic needs of MCT-activated macrophages do dissect new inroads we could manipulate to ultimately boost MCT anticancer immunotherapy. Interestingly, the metabolic requirements of MCT-polarized BMDMs reported here contradict previous reports that correlate increased glycolytic and dysfunctional mitochondrial activity with tumoricidal macrophages and other immune cells[187,188]. MCT-stimulated BMDMs indeed display metabolic features similar to M2 macrophages and clearly distinct to those of prototypical M1 BMDMs. However, both glycolytic dependence and uptake of the

glucose analogue 2NBDG suggest that MCT-stimulated BMDM use glucose to fuel the mitochondrial metabolism, differentiating themselves from M2 and resembling M1 macrophages in substrate usage. Nevertheless, we cannot yet completely exclude a role for fatty acids in the nutrient requirement of MCT-TAM or MCT-BMDM. These data altogether suggest MCT macrophages resemble M2 in mitochondrial dependency and M1 in the metabolic substrate that fuels cellular metabolism. Additional investigations are necessary to elucidate our inconclusive results about the mitochondrial metabolism of MCT BMDMs, considering that they sustain their energetic metabolism through mitochondria but surprisingly present the lowest mitochondrial mass and potential. Mitochondria-intrinsic biology and the activity of its efflux pumps can directly hinder the characterization of mitochondrial metabolism with fluorescent dyes[189]. Methodologies such as electron microscopy and immunofluorescent approaches have revealed accurate to understand the biology and function of mitochondria[189–191] and may as well contribute for the study of MCT BMDMs mitochondrial metabolism.

We believe that this metabolic profile may provide a fitness advantage to the MCT-TAM, which would be less in competition with tumour cells for local nutrients, given that they are more efficient at producing energy from one glucose molecule. Indeed, it may be desirable to have antitumour immune subsets capable to exert their functions in a manner dependent on mitochondrial oxidative metabolism, similarly to protumour immune cells[186]. In line with this, one mechanism responsible to propagate exhaustion in cytotoxic CD8 T cells is the deregulation of the OXPHOS activity and the induction of dysfunctional mitochondria[192]. Correspondingly, it was shown that mitochondrial activation chemicals synergize with anti-PD-1 blockade to promote antitumor T cell activity[193]. Another example is the therapeutic leverage of bezafibrate, that increases OXPHOS capacity and mitochondrial mass to foster the proliferation of naïve T cells, improving effector function in CTLs[194]. It is reasonable that intermediate levels of iNOS expression and subsequent NO production may affect tumour growth and T cell responses without inducing TAM metabolic rewiring and immunosuppression. More studies about quantifying the local NO production and diffusion in the TME[195] are required. On a therapeutical point of view, recent reports have shown that immune cell metabolic manipulation can be achieved for the benefit of the host. For instance, human macrophages conditioned with pancreatic ductal adenocarcinoma cells display a pronounced glycolytic signature, both in a metabolic flux assay and at transcriptome level. This highlights that *in vitro* data may not always efficiently recapitulate *in vivo* conditions. Interestingly, inhibition of glycolysis by inhibiting HK2 with 2-deoxyglucose (2DG), was sufficient to disrupt the protumoural functions of these macrophages[196]. In addition, inhibition of mTOR, which limits

glycolysis, was shown to increase protumoural angiogenic responses[197]. Finally, it is also possible to change functional outcome by providing a given type of nutrient. Fatty acids, for example, synergize with IL-4 to drive M2 polarization and transcriptionally activate PPAR-δ[198], which promotes lipid uptake and storage. PPAR-δ is then crucial for M2-like polarization by directly binding and regulating genes involved in the M2 activation program. Altogether, this literature leads us to propose that drugs capable of activating OXPHOS and upregulating glycolysis may promote the MCT-functional phenotype of TAMs.

Summary

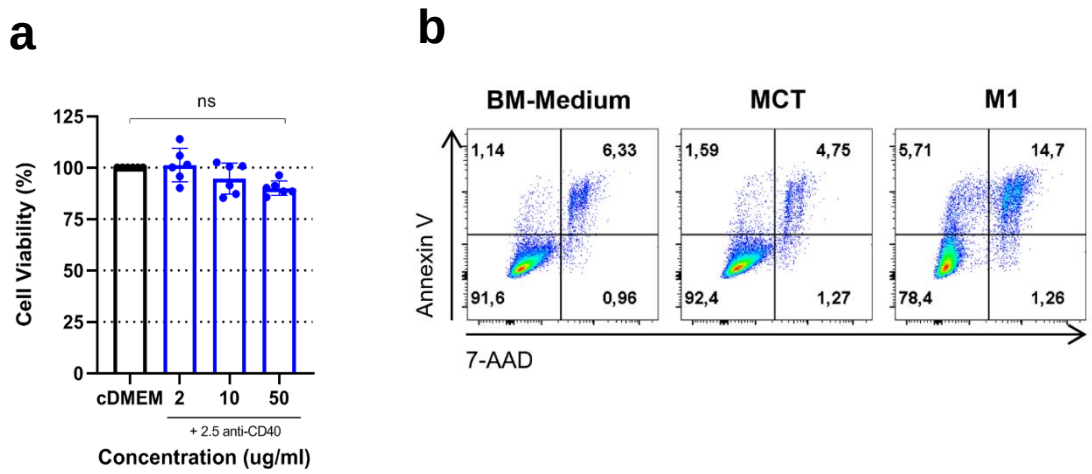
This study indicates MCT effectively induces antitumour macrophages without shifting their metabolism away from mitochondria, as previously reported in antitumour immune cells. Thus, upon treatment, macrophages acquire antitumour functions while retaining a tumour-fitting metabolic profile, suited to the environmental nutrient availability. Furthermore, reduced lactate production by MCT-activated macrophages does not enhance the acidification of the surrounding milieu, maintaining a permissive TME for an antitumour immunologic response, given that lactate can drive tolerogenic and immunosuppressive immune cells[184,185,199]. Emerging studies on the immunometabolism field have demonstrated the metabolic requirements of immune cells in the TME greatly influence the success of immunotherapies[200]. Enhancing immune cell fitness in the tumour bed by metabolic manipulation may have the potential to synergize with existing therapeutic strategies. The provocative data presented here will prompt more studies to unravel if and how mitochondria-centred cellular metabolism regulates MCT-activated macrophage antitumour immunity. We expect to unmask new avenues to enhance MCT immunotherapy and to develop effective combination strategies.

In conclusion, the work presented here proposes that MCT immunotherapy effectively induces iNOS+ antitumour macrophages that drive tumour growth delay and orchestrate long-term cancer elimination, highlighting the antitumour potential displayed by TAMs. Together with our results on the metabolic status of MCT-activated macrophages, this thesis reports the feasibility and efficacy of macrophage-targeted immunotherapies and suggest increasing understanding of immunometabolism within the TME can open up innovative avenues to improve cancer immunotherapy.

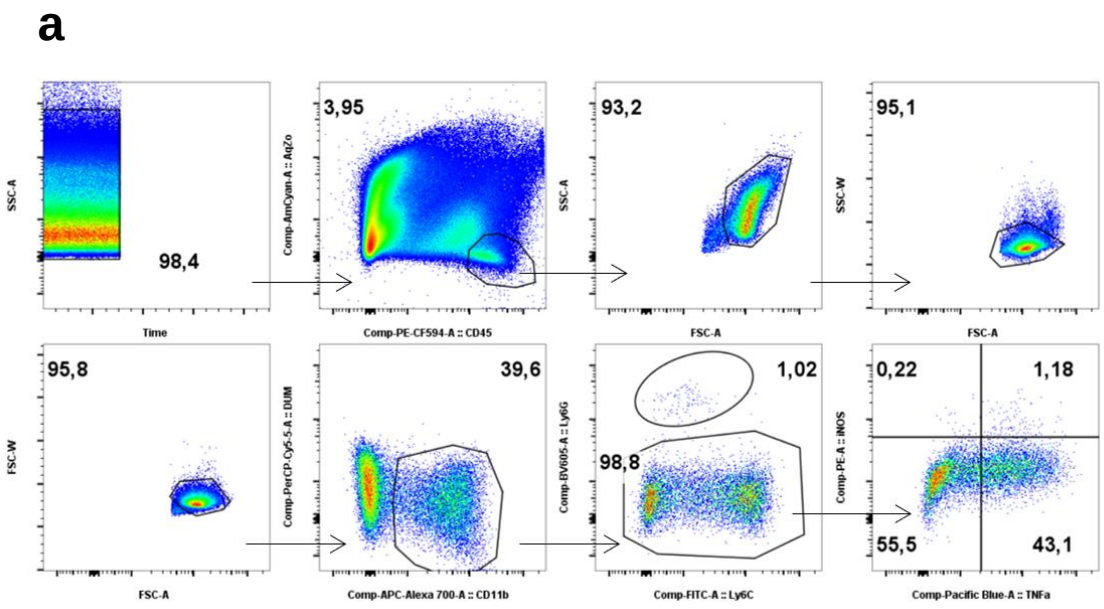
SUPPLEMENTARY INFORMATION

Antibodies for Flow Cytometry				
Antibody	Manufacturer	Clone	*In vitro*	*In vivo*
FITC Anti-mouse Ly6C	Invitrogen	HK1.4		**X**
PE Anti-mouse iNOS	Invitrogen	CXNFT	**X**	**X**
PE-Cy7 Anti-mouse F4/80	Invitrogen	BM8	**X**	**X**
PE/Dazzle™ Anti-mouse CD45	BioLegend	30-F11		**X**
Alexa700 Anti-mouse CD11b	BioLegend	M1/70	**X**	
APC-Cy7 Anti-mouse NK1.1	BioLegend	PK136		**X**
APC-Cy7 Anti-mouse CD3e	Invitrogen	17A2		**X**
APC-Cy7 Anti-mouse CD19	BioLegend	6D5		**X**
Pac-Blue Anti-mouse TNFα	Invitrogen	MP6-XT22		**X**
Amcyan Anti-mouse Zombie Aqua	BioLegend		**X**	**X**
BV605 Anti-mouse Ly6C	BioLegend	HK1.4	**X**	**X**
BV605 Anti-mouse Ly6G	BioLegend	1A8		**X**
BV711 Anti-mouse CD11b	BioLegend	M1/70	**X**	

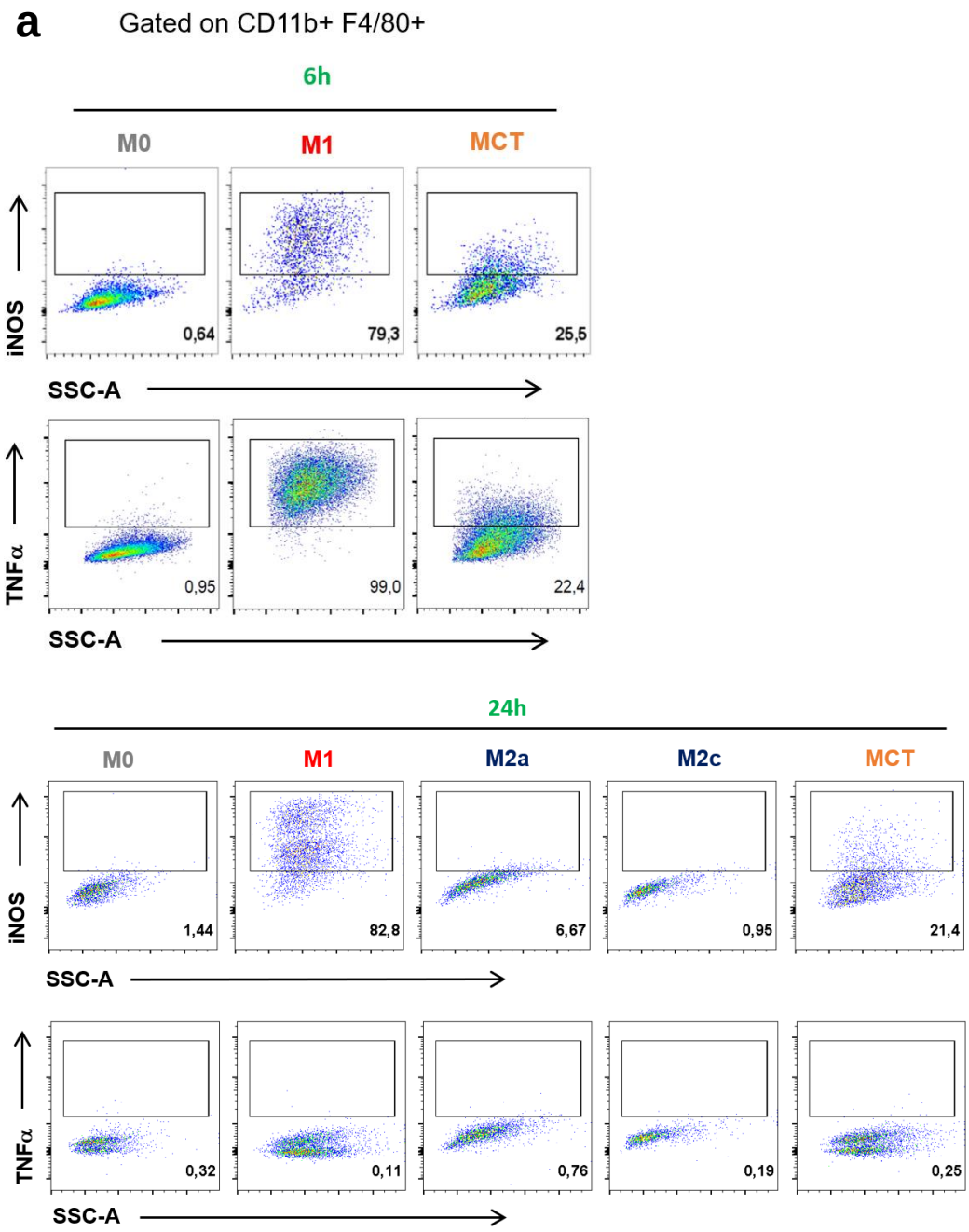
Supplementary table 1 – List of antibody clones and respective manufacturer used in flow cytometry analysis.



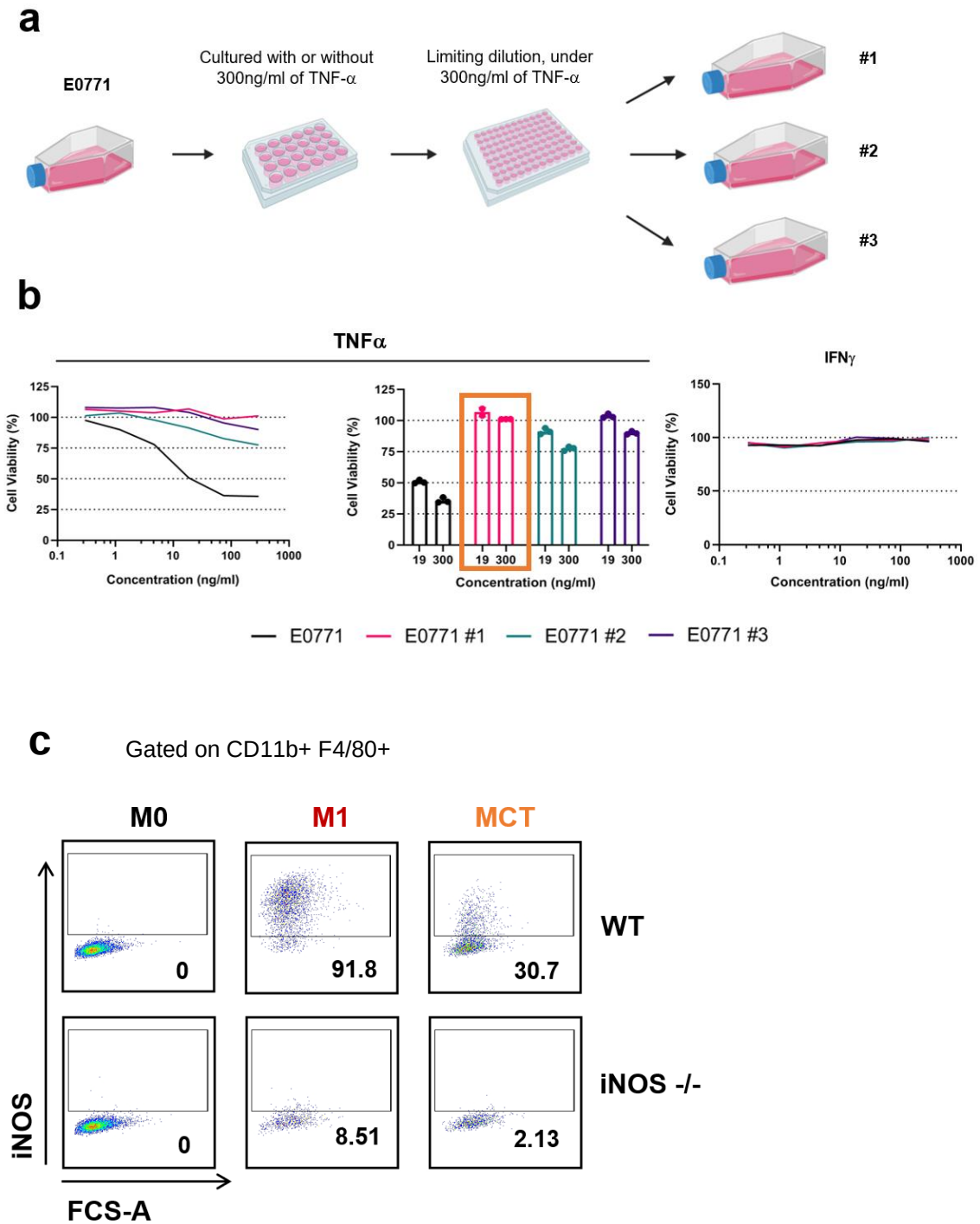
Supplementary figure 1 – Assessment of E0771 Sensitivity to TLR Ligands and Recombinant Cytokines. a, E0771 cell viability after incubation with MCT (increasing doses of TLR3 ligand with a fixed dose of anti-CD40) for 48h. Data from two independent experiments. Significance was calculated with Kruskal–Wallis test followed by post-hoc Dunn's test**. b,** Flow cytometry of E0771 cells cultured with BM-medium (control), MCT or M1 BMDM-SN for 48h. Tumour cells were analysed with Annexin V and 7-AAD stainings to detect apoptotic and necrotic cells, respectively.



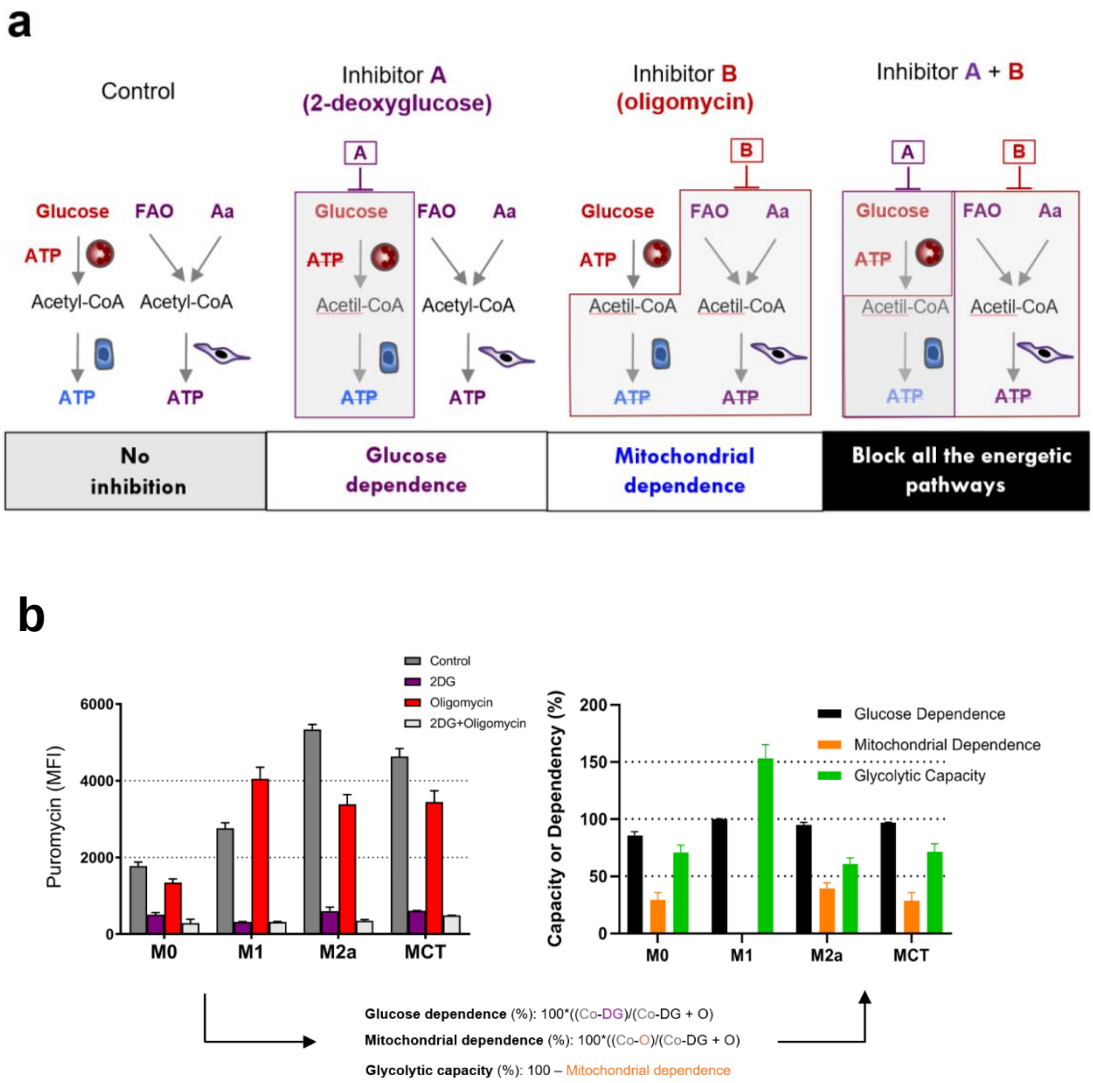
Supplementary figure 2 – Gating strategy to analyse tumour-infiltrating macrophages. Representative gating strategy to analyse CD45+ CD3- CD19- NK1.1- Ly6G- CD11b+ Ly6c+/- macrophages from harvested tumours.



Supplementary figure 3 – Myeloid Cell-Treatment induces an antitumoral-like phenotype in BMDMs. a, flow cytometry data of CD11b+ F4/80+ bone marrow-derived macrophages (BMDMs) after 6h (top) and 24h of polarization (bottom). Data representative of several independent experiments.



Supplementary figure 4 – Methodologies to dissect MCT macrophage antitumour activities. a, Schematic representation of the protocol to isolate the E0771 TNFα-resistant clones. E0771 cells were cultured with 300ng/ml of TNF-α for three weeks. Fresh TNFα was replenished every other day. A limiting dilution was performed to isolate single cell clones from the parental E0771 cells proliferating in the presence of TNFα. The 3 most proliferating clones were expanded and further subjected to a Cell Viability Assay, shown in **b, left panel**, to confirm acquired TNFα resistance. We confirmed E0771 TNFα-resistant clones did not acquire sensitivity to IFNγ **(b, right panel)**. **c,** flow cytometry data of WT and iNOS-ko BMDMs after 24h of *in vitro* polarization to confirm the absence of iNOS production in the knock-out mice.



Supplementary Figure 5 – Scenith methodology to profile metabolism at the single-cell level by flow cytometry. a, Scenith assesses the impact of metabolic inhibitors on protein synthesis. Mean fluorescence intensity (MFI) of Puromycin is analysed in each condition (Control: no inhibition; 2-DG: inhibition of glycolysis; Oligomycin: inhibition of OXPHOS and 2-DG + Oligomycin: inhibition of all energetic pathways). **b**, calculation of glucose dependence, mitochondrial dependence and glycolytic capacity (right panel) based on puromycin MFIs (left panel). More details are depicted in the Methods section.

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