UNIVERSIDADE DE LISBOA
Faculdade de Medicina

# DISSECTING FUNCTIONAL AND MOLECULAR SIGNATURES OF ANTI-TUMOUR MYELOID CELLS *IN VIVO*

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Supervisor: Karine Serre, PhD
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Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

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A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 19 de Fevereiro de 2019.

"Nothing in life is to be feared, it is only to be understood.
Now is time to understand more, so that we may fear less"
- Marie Curie

Ao meu maior fã,

Avô

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# AGRADECIMENTOS

Porque acredito que este trabalho é mais um passo na minha vida profissional, dedico-o a todos aqueles que, de uma forma directa ou indirecta, estiveram envolvidos na concretização desta tese de mestrado e que sem eles nada disto seria possível.

Em primeiro lugar quero agradecer ao Professor Bruno Silva-Santos pela confiança, entusiasmo e pela oportunidade de poder aprender com os melhores. Agradeço também a todos os elementos do laboratório, sem excepção, pela boa disposição, entreajuda e discussões científicas. Um muito obrigada à Natacha pela paciência por lhe estar sempre a interromper para fazer perguntas "administrativas".

Um agradecimento muito especial a toda a equipa que trabalhou directamente comigo. À Karine agradeço-lhe toda a confiança que depositou em mim para desenvolver este projecto, por todo o conhecimento transmitido e por toda a paciência ao longo deste último ano. Obrigada pela disponibilidade e sinceridade, mas principalmente pela oportunidade que me deu de errar, de aprender e de crescer como cientista. Quero igualmente agradecer à Sofia por toda a ajuda na execução experimental, disponibilidade, simpatia e acima de tudo por todos os conselhos. Um grande obrigada às duas por tudo o que me ensinaram, quer na ciência, quer para a vida.

Como é óbvio também não poderia deixar de agradecer aos meus dois companheiros, não só de mestrado, mas também de limpeza da sala de cultura: Tiago e Daniel. A eles junto também o Carlos e o Henning a quem  agradeço todas as conversas e discussões científicas. Um obrigada também muito especial ao Miguel pelas nossas conversas e dados partilhados para esta tese.

Quero ainda aproveitar para agradecer aos elementos vizinhos dos laboratórios do Professor Luís Graça e Marc Veldhoen pelos protocolos, reagentes e simpatia. Um obrigada também ao laboratório do Professor Luís Costa, mais especificamente à Professora Sandra Casimiro, pelos reagentes necessários para optimizar a técnica de imunohistoquímica.

Gostaria também de agradecer a todos os membros das unidades que utilizei ao longo deste último ano por toda a simpatia e disponibilidade: biotério e citometria de fluxo. Um obrigada muito especial a todos os elementos da unidade de Histologia com quem passei os últimos meses: Tânia, Ana Rita, Pedro e Vitor. Obrigada por todo o apoio, dedicação e discussões. Posso dizer que o vosso "Livro das respostas" também foi um grande incentivo e ajuda nos momentos mais difíceis.

Um muito obrigado aos meus dois pilares, Sara e Henrique, não só por todas as palavras de encorajamento, força e ânimo que me deram, mas também pela vossa amizade e pelas conversas descontraídas. Um obrigada também muito

especial a todos os meus colegas do Pavilhão do Conhecimento por todo o apoio, partilha de ideias e discussões científicas durante este último ano.

Por fim, mas não menos importante, um enorme obrigada à minha família, sem a qual esta etapa não teria sido concretizada. Aos meus avós, tios e primos por acreditarem em mim, vibrarem com os meus feitos e por se orgulharem como orgulham. À minha mãe, cuja disponibilidade é total quando se trata de me apoiar e que fez com que todas as minhas preocupações se transformassem em resoluções. Ao meu pai, que apesar de não demonstrar o que sente, quero agradecer-lhe por todo o apoio e acima de tudo pela liberdade de escolha, pois nunca foi capaz de pôr em causa o caminho que quis seguir, mesmo que ás vezes não pareça ser o mais estável: obrigada por me proporcionares chegar até aqui nos meus estudos.

Ao Lívio, só me resta agradecer-lhe por todo o seu amor, suporte e felicidade partilhada ao longo destes oito anos. Obrigada pela compreensão, por ouvires as minhas preocupações e desabafos, mas acima de tudo por toda a paciência que tens para o meu mau feitio e humor!

Foi efectivamente um ano cheio de obstáculos e muito intenso, mas tornou-se muito mais fácil de ultrapassar tendo pessoas como vocês para superar todas as dificuldades. E apesar de este trabalho ter o meu nome, tem uma pitada de todos vocês e muito pouco de só meu. A todos vocês: Muito obrigada/ Thank you/ Merci beaucoup/ Grazie mille/ Muchas gracias / Brigadão (com sotaque brasileiro)/ Vielen Dank!

# ABBREVIATION LIST

| | |
|---|---|
| ACT | Adoptive cell transfer |
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| ADCP | Antibody-dependent cell-mediated phagocytosis |
| ALL | Acute lymphocytic leukemia |
| AML | Acute myelogenous leukemia |
| AP | Alkaline phosphatase |
| APC | Antigen-presenting cell |
| A2aR | Adenosine 2a receptor |
| BCG | Bacillus-Calmette Guerin |
| BMDM | Bone marrow -derived macrophages |
| BLBC | Basal-like breast cancer |
| BTLA | B and T cell lymphocyte attenuator |
| B7-H3 | B7 homolog 3 |
| CAR | Chimeric antigen receptor |
| CCL | C-C motif chemokine ligand |
| CCR | C-C motif chemokine receptor |
| cDNA | Complementary DNA |
| CLL | Chronic lymphocytic leukemia |
| CSF1 | Colony-stimulating factor 1 |
| CSF1R | Colony-stimulating factor 1 receptor |
| CTL | Cytotoxic T Lymphocytes |
| CTLA-4 | Cytotoxic T lymphocyte antigen 4 |
| CTV | Cell track violet |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| DMEM | Dulbelcco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ER | Estrogen receptor |
| Fas | First apoptosis signal |
| FasL | First apoptosis signal ligand |
| FCS | Fetal Calf Serum |
| FDA | U.S. Food and Drug Administration |
| FMO | Fluorescence minus one |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| gp100 | Glycoprotein 100 |
| HBV | Hepatitis B virus |

| | |
|---|---|
| HCC | Hepatocellular carcinoma |
| HER2+ | Human epidermal growth factor receptor 2 |
| HIF | Hypoxia-inducible factor |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papillomavirus |
| HRG | Histidine-rich glycoprotein |
| HRP | Horseradish peroxidase |
| HSC | Hematopoietic stem cell |
| IC | Immune complex |
| ICI | Immune checkpoint inhibitor |
| IFN | Interferon |
| IFN-$\gamma$ | Interferon-$\gamma$ |
| IHC | Immunohistochemistry |
| IL | Interleukine |
| ILC | Innate lymphoid cell |
| IMM\|JLA | Instituto de Medicina Molecular \| João Lobo Antunes |
| iNOS | Inducible nitric oxide sythase |
| IP | Intraperitoneal |
| IT | Intratumoural |
| IV | Intravenous |
| LAG-3 | Lymphocyte activation gene-3 |
| M1 | Classically activated macrophages |
| M2 | Alternatively activated macrophages |
| mAb | Monoclonal antibody |
| MAGE-A3 | Melanoma-associated antigen 3 |
| MART-1 | Melanoma-associated antigen recognized by T cells |
| MCP-1 | Monocyte chemoattractant protein-1 |
| mCRPC | Metastatic castration-resistant prostate cancer |
| M-CSF | Macrophage colony-stimulating factor |
| M-CSFR | Macrophage colony-stimulating factor receptor |
| MDSC | Myeloid-derived suppressor cell |
| MEM NEAA | Minimum Essential Medium Non-Essential Amino Acids |
| MHC | Major histocompability complex |
| M-MLV | Moloney Murine Leukemia Virus |
| MMP | Metalloproteinase |
| MPO | Myeloperoxidase |
| miRNA | Micro-RNA |
| NET | Neutrophil extracellular traps |
| NHL | Non-Hodgkin's lymphoma |
| NO | Nitric Oxide |
| NSCLC | Non-small cell lung cancer |
| NY-ESO-1 | New York esophageal squamous cell carcinoma |

| | |
|---|---|
| OS | Overall survival |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate Buffer Saline |
| PD-1 | Programmed cell death protein 1 |
| PDGF | Platelet-derived growth factor |
| PD-L1 | Programmed cell death protein ligand 1 |
| PMA | Phorbol 12-myristate 13-acetate |
| PR | Progesterone receptor |
| PRR | Pattern recognition receptor |
| PSA | Prostatic acid phosphatase |
| P/S | Penicillin/Streptomycin |
| RBC | Red Blood Cell |
| Rag2$^{-/-}\gamma$c$^{-/-}$ | Rag2$^{-/-}$ common gamma chain |
| RCC | Renal cell carcinoma |
| ROS | Reactive oxygen species |
| rpm | Rotations per minute |
| RT | Room temperature |
| scFv | Single-chain fragment variant |
| SIRP$\alpha$ | Signal regulatory protein $\alpha$ |
| sVEGF | Soluble vascular endothelial growth factor |
| TAA | Tumour-associated antigen |
| TAM | Tumour-associated macrophages |
| TAN | Tumour-associated neutrophils |
| TCR | T cell receptor |
| TIL | Tumour-infiltrating lymphocytes |
| TIM-3 | T cell immunoglobulin-3 |
| TGF-$\beta$ | Transforming growth factor $\beta$ |
| Th1 | T helper type 1 cell |
| Th2 | T helper type 2 cell |
| TLR | Toll-like receptor |
| TME | Tumour microenvironemt |
| TNBC | Triple-negative breast cancer |
| TNF-$\alpha$ | Tumour necrosis factor $\alpha$ |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Treg | Regulatory T cell |
| TSA | Tumour-specific antigen |
| TSLP | Thymic stromal lymphopoiectin |
| UC | Urothelial carcinoma |
| VEGF | Vascular endothelial growth factor |
| VISTA | V-domain Ig suppressor of T cell activation |
| WT | Wild-type |

# ABSTRACT

The tumour microenvironment is a heterogeneous ecosystem that can be densely populated by immune cells, both of lymphoid and myeloid origin. Usually, up to 50% of the immune infiltrate can be constituted by a multitude of myeloid cells that, accordingly to environmental cues, can either act as anti-tumoural or pro-tumoural effector cells.

Plasticity is a widely accepted hallmark of myeloid cells and to date there has been a disproportionate attention on pro-tumour over anti-tumour myeloid cell functions. However, we believe that more efforts should be put on understanding how to enhance the protective activity of myeloid cells in cancer. In this thesis, to study myeloid cells as they perform anti-tumour effector functions *in vivo*, we took advantage of their inherent capacity to respond to maturing agents such as TLR ligands and co-stimulatory agonists.

We found that in the orthotopic E0771 mammary tumour model, the injection of TLR3 ligand plus anti-CD40 mAb was able to induce tumour regression in a macrophage-dependent manner. These macrophages were stimulated to produce specific pro-inflammatory cytokines and enzymes. In addition, CD8+ T cells were activated, thus promoting a cytotoxic response leading to the complete eradication of tumours in TLR3/anti-CD40 treated mice.

These results led us to question whether other distinct TLR ligands could induce complete remission of the tumour, potentially dependent on different anti-tumour myeloid cell lineages. Indeed, we found that TLR4 ligand in combination with anti-CD40 mAb induced tumour elimination in a neutrophil-dependent manner.

Altogether, in this thesis we were successfully able to shape the tumour microenvironment towards boosting the anti-tumour potential of macrophages and neutrophils using different and non-overlapping treatments.

In sum, our results lay the groundwork for further studies that will combine unbiased approaches (transcriptomics) with *in situ* assessment of the biology, functionality and differentiation program of anti-tumour macrophages and neutrophils. In the longer run, we hope such knowledge will help to design new strategies to manipulate myeloid cells in order to unleash their anti-tumour potential and thus contribute to more efficient cancer immunotherapies.

**Keywords:** macrophages, neutrophils, TLR ligands, anti-CD40 agonist mAb, cancer immunotherapy

# RESUMO

Ao longo dos últimos anos, o aumento do conhecimento acerca do sistema imunitário e da sua importância na destruição das células tumorais tem levado ao desenvolvimento de novas estratégias imunoterapêuticas. Estas estratégias representam uma nova era na área da oncologia e têm como principal objectivo estimular e potenciar uma resposta imune. Porém, apesar de todos os resultados clínicos notáveis, uma fração significativa dos pacientes não responde à imunoterapia. Esta resistência é o resultado da capacidade das células tumorais manipularem o microambiente e promoverem imunossupressão local permitindo-lhes escapar ao reconhecimento e eliminação por células imunes.

O microambiente tumoral tem sido alvo de grande atenção por parte dos investigadores por estar directamente envolvido na progressão tumoral. Para além das células neoplásicas, este é também constituído por diversas populações celulares, tais como as do estroma ou das linhagens linfóide e mielóide do sistema imunitário. Habitualmente, mais de 50% da infiltrado imune é composto por células mielóides que, de acordo com as informações que recebem do microambiente, podem estimular ou inibir a resposta imunitária anti-tumoral.

As células mielóides têm uma grande plasticidade e até hoje tem havido uma atenção desproporcional entre as células que promovem o crescimento do tumor (pró-tumorais) relativamente às que tentam travar o seu crescimento (anti-tumorais). Contudo, acreditamos que deveriam ser feitos mais esforços de modo a perceber de que forma poderíamos aumentar a actividade protectora destas células. Para isso, neste trabalho utilizou-se um modelo ortotópico de células tumorais mamárias de ratinho (E0771) com o objectivo de estudar a actividade anti-tumoral das células mielóides durante o desenvolvimento tumoral *in vivo*, tirando partido do facto de estas serem capazes de responder a vários sinais, mais especificamente a ligandos de receptores de Toll (TLR) e moléculas co-estimuladoras.

Nesta tese foi possível demonstrar que *in vivo*, a injecção intra-tumoral dos ligandos de TLR2/1 (Pam3CSK4), 2/6 (Pam2CSK4), 3 (Poly I:C), 4 (LPS) e 9 (CpG) em combinação com o agonista anti-CD40, levou à remissão total do tumor. Além disso, *in vitro* estes ligandos de TLR não têm um impacto directo na proliferação das células tumorais, o que significa que o efeito destes tratamentos deverá ocorrer através da manipulação das células células que constituem o microambiente tumoral.

O Poly I:C é uma molécula de RNA de cadeia dupla associada a infeções virais e que consegue ser muito semelhante à activação que é feita aquando do aparecimento de um tumor. *In vitro* foi ainda possível observar que este imunoestimulador, em combinação com o anticorpo agonista anti-CD40, é um dos que menos afecta a expressão de moléculas do complexo maior de imunohistocompatibilidade classe I e II bem como dos marcadores

imunossupressores dos ligandos de PD-1 das células tumorais. Com isto, decidimos dissecar os mecanismos específicos de acção pelos quais o tratamento intra-tumoral com o Poly I:C em combinação com o anticorpo agonista anti-CD40 levavam à eliminação do tumor. Desta análise, concluiu-se o efeito era dependente dos macrófagos (CD11b+ F4/80+ Ly6C+/-), uma vez que após a depleção destas células o tratamento deixou de funcionar. Descobrimos ainda que o tratamento levou a um aumento da produção de citocinas pró-inflamatórias, tais como IL-1$\beta$ e TNF-$\alpha$ ou mesmo enzimas, como o iNOS pelos macrófagos. Paralelamente a este aumento, houve uma diminuição da expressão do marcador imunossupressor PD-L1 nos ratinhos que tinham sido tratados em comparação com os não tratados.

Os nossos resultados mostraram ainda que, após a re-injecção das células tumorais E0771 na glândula mamária de ratinhos onde o tumor já tinha sido eliminado, este não voltava a crescer. Este resultado sugere assim a capacidade de este tratamento com o ligando do TLR3 em combinação com o agonista anti-CD40 criar uma memória imunológica capaz de reconhecer o tumor como estranho e eliminá-lo. Sabendo que as células T CD8+ estão associadas à memória imunológica e são altamente citotóxicas, colocámos então a possibilidade de estas terem também um papel na eliminação do tumor uma vez que a produção de TNF-$\alpha$ e IFN-$\gamma$ por estas células era aumentada pelo tratamento. As células T CD8+ mostraram então ser fundamentais para a eliminação o tumor, sendo que a sua depleção evitava a regressão tumoral.
 dependente das células T CD8+. Além disso, as células T CD8+, cuja produção de TNF-$\alpha$ e IFN-$\gamma$ é aumentada pelo tratamento, mostraram ser fundamentais para a eliminação do tumor, uma vez que a sua depleção evitava a regressão tumoral.

Através destas descobertas foi então possível construir um modelo dividido em dois passos (*two-step model*) onde o tratamento com o ligando de TLR3 em combinação com o anticorpo agonista anti-CD40 leva à activação dos macrófagos e mais tarde, de uma forma directa ou indirecta (via células dendríticas), as células T CD8+ são também activadas e tornam-se capazes de gerar uma resposta citotóxica contra os tumores, levando à sua total erradicação nos animais tratados.

Posteriormente analisámos a acção de outros ligandos de TLR na capacidade de indução de remissão total do tumor e de activação de outras células com propriedades propriedades anti-tumorais da linhagem mielóide: os neutrófilos. Chegámos à conclusão de que, em animais onde era feita a depleção destas células através do anticorpo anti-Gr1, a injecção intra-tumoral do Pam3CSK4, Pam2CSK4 e CpG  com o anticorpo agonista anti-CD40 continuava a gerar uma resposta anti-tumoral resultando na regressão do tumor. Porém,  com a injecção intra-tumoral do ligando de TLR4 em combinação com o agonista anti-CD40, em ratinhos depletados para neurófilos mostrou-se ineficaz na indução de regressão tumoral. Estes resultados levaram-nos a concluir que a injecção do ligando TLR4 com o anticorpo agonista anti-CD40 não só levava à regressão total

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do tumor, mas também actuava directamente nos neutrófilos, tornando-os anti-tumorais. No entanto, são necessários estudos mais detalhados para perceber de que forma é que este tratamento funciona tal como foi feito para os macrófagos através do tratamento com o Poly I:C em combinação com o anticorpo anti-CD40.

Neste projecto fomos então capazes de alterar o microambiente tumoral e, em particular, de estimular a capacidade anti-tumoral dos macrófagos e dos neutrófilos através de diferentes tratamentos.

Em suma, os resultados nesta tese são promissores e levam-nos a ambicionar por mais estudos que sejam capazes de decifrar quais os programas de transcrição e diferenciação responsáveis por tornar estas células imunes anti-tumorais. No futuro, acreditamos que estes resultados nos poderão ajudar a desenvolver novas estratégias para manipular e aumentar o poder anti-tumoral destas células mielóides, de modo a melhorar a eficácia da imunoterapia do cancro.

**Palavras-chave:** macrófagos, neutrófilos, ligandos de TLR, agonista anti-CD40, imunoterapia do cancro

# CHAPTER 1: INTRODUCTION

## 1. The Immune System

Despite being surrounded by harmful organisms, toxins and the threat of our own transformed cells, humans manage to survive largely because of our immune system. The principal challenge for the host is to detect the pathogens or transformed cells and mount a rapid defense response. This response is classically divided into two components accordingly to the speed and specificity of the reaction: the innate or natural and adaptive or acquired immunity (Beutler, 2004)(Figure 1).

## 1.1. Innate and adaptive arms of the immunity

Innate immune response includes not only chemical barriers, such as lysozyme in tears or low pH in the stomach, but also physical barriers like the epithelium in the skin and cilia in airways in order to keep invaders out. In vertebrates, the innate immune response is the first line of defense against infectious disease and is characterized by a quick response that is executed in part through the expression of pattern recognition receptors (PRRs), which are fundamental for the recognition of a wide range of pathogens. Such PRRs include NOD-like receptors or C-type lectin receptors, but toll-like receptors (TLRs) were the first described and have been extremely studied as possible targets in several types of diseases, like cancer. This particular interest for TLRs comes from the fact that they are fundamental sensor molecules involved in the recognition of pathogen associated molecular patterns (PAMPs) and initiate a signaling cascade that leads to the expression of several inflammatory cytokines and chemokines necessary to arm the host cell against the pathogen (Kawai & Akira, 2011; Medzhitov & Janeway, 2002). This natural system is largely composed and dependent upon myeloid cells: professional immunocytes that are able to engulf and destroy pathogens (monocytes/macrophages, neutrophils, and dendritic cells (DCs)), and cells that produce inflammatory mediators (basophils, eosinophils and mast cells) (Tonegawa, 1983).

The adaptive arm is constituted by lymphocytes: B and T cells (Burnet, 1958). B cells develop in the bone marrow and upon stimulation, secrete antibodies that are able to remember previous antigen encounters and quickly eliminate extracellular microorganisms (Delves & Roitt, 2000). T cells originate from the thymus, and may further be divided in $\alpha\beta$ T cells (large subset) and $\gamma\delta$ T cells, according to the chains that constitute their T cell receptor (TCR). $\alpha\beta$ T cells can be segregated into helper CD4+ T cells and cytotoxic CD8+ T cells. These cells circulate and are activated in secondary lymphoid organs by antigen-presenting cells (APCs) that process antigens and display specific cell proteins at their surface, known as major histocompability complex (MHC) (Cooper & Alder,

2006). Regarding helper CD4+ T cells, they can be divided into three major subtypes with distinct phenotypes: T helper type-1 (Th1), T helper type-2 (Th2) and T helper type-17 (Th17) cells. Th1 cells secrete interferon (IFN)-$\gamma$ and tumour necrosis factor (TNF)-$\beta$, which make these cells highly anti-inflammatory. On the other hand, Th2 cells secrete several interleukins (ILs), such as IL-4 or IL-10 and are not only involved in the elimination of extracellular parasites, but also on the activation of B cells. Finally, Th17 cells secrete IL-6, IL-17, IL-22 and TNF-$\alpha$. They are involved in tissue inflammation, autoimmunity and in the combat of extracellular bacteria and fungi. Regulatory T cells (Tregs) also derive from naive CD4+ T cells and they secrete IL-10 and transforming growth factor (TGF)-$\beta$. They are immunosuppressive and are involved in the regulation of immune responses, maintenance of tolerance to self-antigens and prevention of autoimmune diseases (Kaiko, Horvat, Beagley, & Hansbro, 2007).

By contrast to $\alpha\beta$ T cells, most $\gamma\delta$ T cells populate epithelial tissues where they quickly respond to stress signals expressed by local cells (Hayday, 2009).

T cell activation is complex, requiring cognate antigen recognition and activation signals from professional APCs such as appropriate costimulatory molecules (CD80 and CD86) and cytokine signaling. Upon activation, T cells proliferate and differentiate into specific effectors. This is regulated by the induction of selective transcriptional programs that lead to the acquisition of effector functions characteristic of CD4+ or CD8+ T cells. For instance CD4 helper T cells usually produce cytokines while CD8+ cytotoxic T cells express effector molecules such as perforin and granzymes (Nicholson, 2016).

Importantly, innate responses occur to the same extend independently of the number of times a pathogen is encountered, while the adaptive system is able to mount a stronger and more specific response during second challenges (Elves, Roitt, Mackay, & Rosen, 2000).

Cells of the adaptive immune system are dependent upon the myeloid compartment: without antigen presentation by innate immune cells (e.g. DCs), and without production of cytokines from innate origin (IL-12, type I IFNs and TNF), adaptive immune responses are not induced. With this in mind, we can say that while adaptive and innate immunity work hand in hand, adaptive immunity depends on innate immunity. This report aims at understanding the role of myeloid cells in cancer. Therefore, we will pay particular attention onwards to the detailed introduction of the myeloid compartment.

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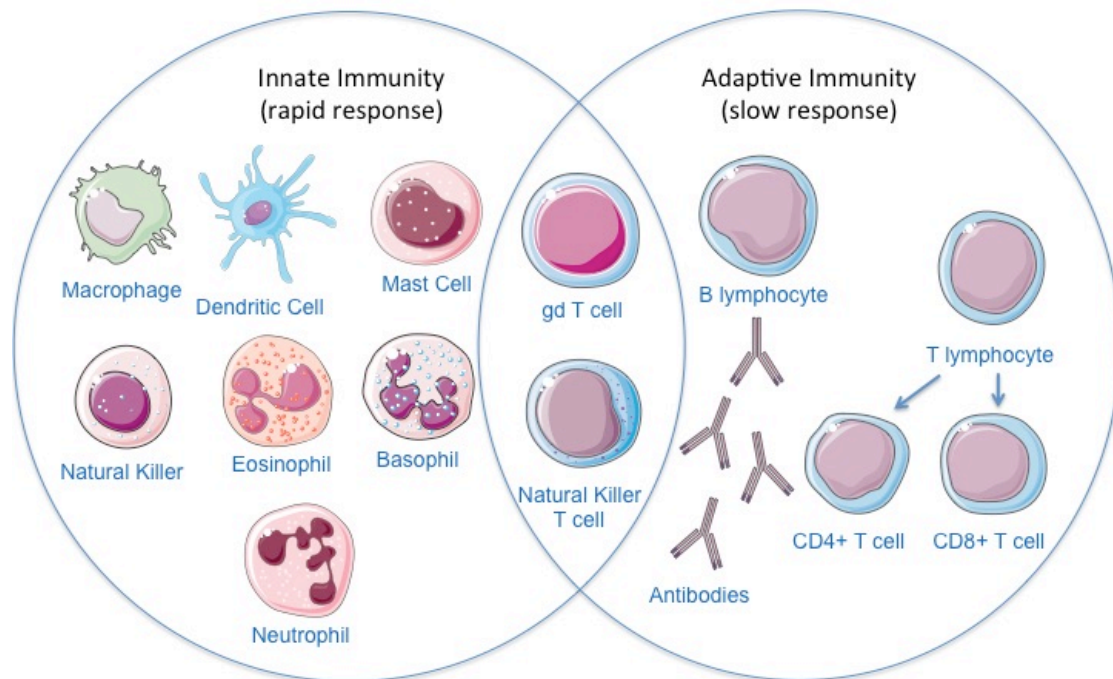
**Figure 1: Cells from innate and adaptive immune systems.** Based on Dranoff, 2004.

## 1.2. Myeloid cells - Ontogeny and Functions

Myeloid cells, that arise from pluripotent hematopoietic stem cells (HSCs) in the bone marrow, are specialized "sentinels" and fighters of the innate immune system, being of key importance in the containment of infection.

Myeloid cells are the most abundant nucleated hematopoietic cells in the human body, are extremely short-lived (usually, less than three days) and are composed of monocytes/macrophages, DCs, and granulocytes (neutrophils, eosinophils, basophils and mast cells) (Gabrilovich, Ostrand-rosenberg, & Bronte, 2012) (Figure 2).

**Figure 2: Hematopoiesis under normal physiological conditions.** Based on Gabrilovich, Ostrand-rosenberg, & Bronte, 2012.

## 1.2.1. Monocytes

During steady state, circulating monocytes have a half-life of one to three days and constitute ~5-10% of peripheral circulation leukocytes in humans (van Furth & Cohn, 1968; Gordon & Taylor, 2005; Yona et al., 2013). These cells are equipped with a set of TLRs, which makes them able to recognize and remove microorganisms, lipids and dying cells by phagocytosis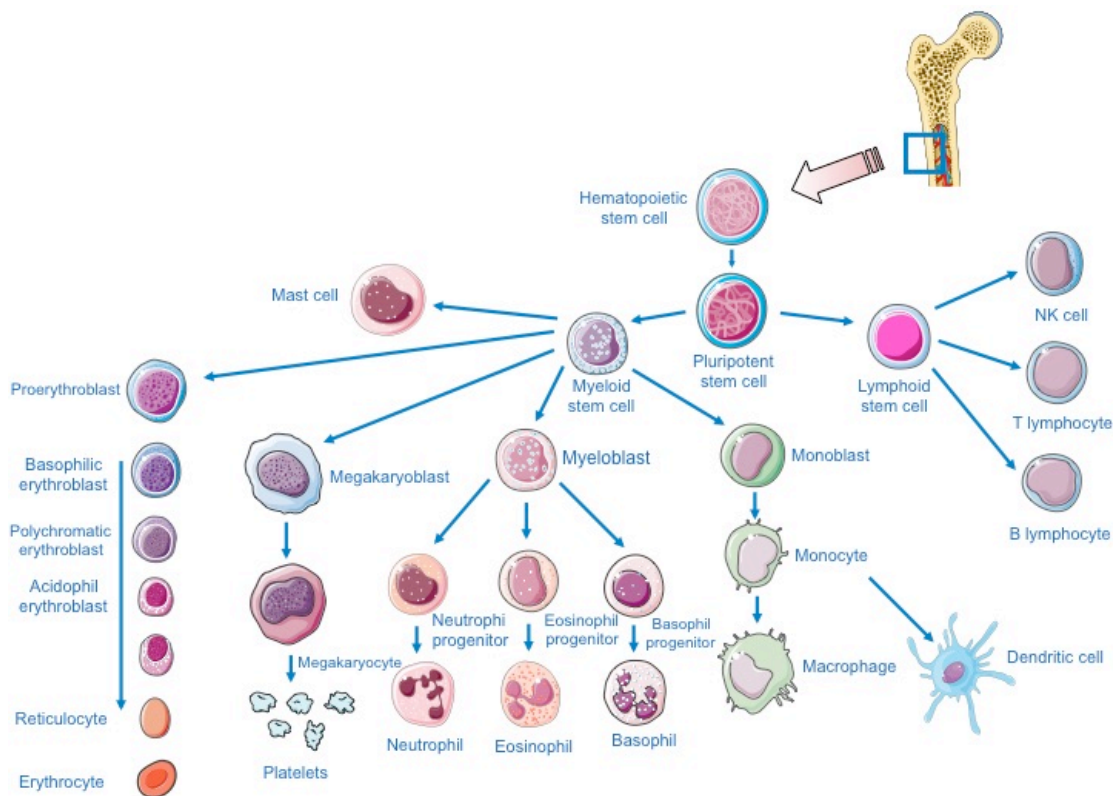. Upon danger signals, monocytes are rapidly recruited to the tissue, where they can differentiate into anti-inflammatory macrophages or dendritic cells (Nahrendorf et al., 2007).

Therefore, these cells are highly plastic, being able to change their functional phenotype accordingly to the stimulus received from the environment.

Studies already have shown that mice deficient in colony-stimulating factor 1 (CSF1) or its receptor, CSF1R are very important for both monocyte differentiation and survival (Dai et al., 2002).

## 1.2.2. Macrophages

Historically, macrophages were first identified in the late 19th century by Élie Metchnikoff because of their phagocytic nature (Metchnikoff, 1892). This discovery gave him Noble Prize in Physiology or Medicine in 1908 together with Paul Ehrlich.

Ontogenically, tissue-resident macrophages derive from at least three

different sources: the embryonic yolk sac, the fetal liver and the bone marrow. During embryogenesis, the first organ to be populated is the brain with macrophages that arise from the yolk sac: microglia. Later on, the fetal liver is populated by macrophages derived from the yolk sac and they give rise to tissue-resident macrophages in several organs, such as bone, lung, liver, spleen, pancreas and kidney (osteoclasts, alveolar, kupffer, splenic, pancreas and kidney macrophages, respectively) and persist into adulthood (Ginhoux & Guilliams, 2016; Guerriero, 2018) (Figure 3). Upon homeostatic changes, such as infections, cardiovascular diseases, obesity (metabolic imbalance) or cancer, bone marrow- derived macrophages also play a crucial role in inflammatory and reparatory responses during pathogenic infection and tissue injury (Cassado, 2015; Mosser & Edwards, 2008; J. Yang, Zhang, Yu, Yang, & Wang, 2014).

As soon as a pathogen enters in the host by any route, macrophages that are embedded in all tissues will be rapidly attracted to the invasive organism (Beutler, 2004). They are capable of engulfing and killing pathogens and throughout the secretion of chemotactic cytokines, they can also recruit other innate and adaptive cells to the site of infection (e.g. neutrophils or T cells, respectively). Even though pathogen detection and phagocytosis can provide the initial stimulus, the activity of macrophages can be increased by cytokines released by CD4+ T cells, such as interferon gamma (IFN-$\gamma$), which is one of the most potent macrophage activator (Duque & Descoteaux, 2014).

Macrophages, although not as efficient as DCs, are also APCs. They are able to initiate an adaptive immune response to most pathogens by presenting antigens to CD4+ T cells via MHC II (Beutler, 2004).

They are highly heterogeneous, which means that they can adapt to distinct tissue environments and are also able to develop niche-specific functions (Cassado, 2015).
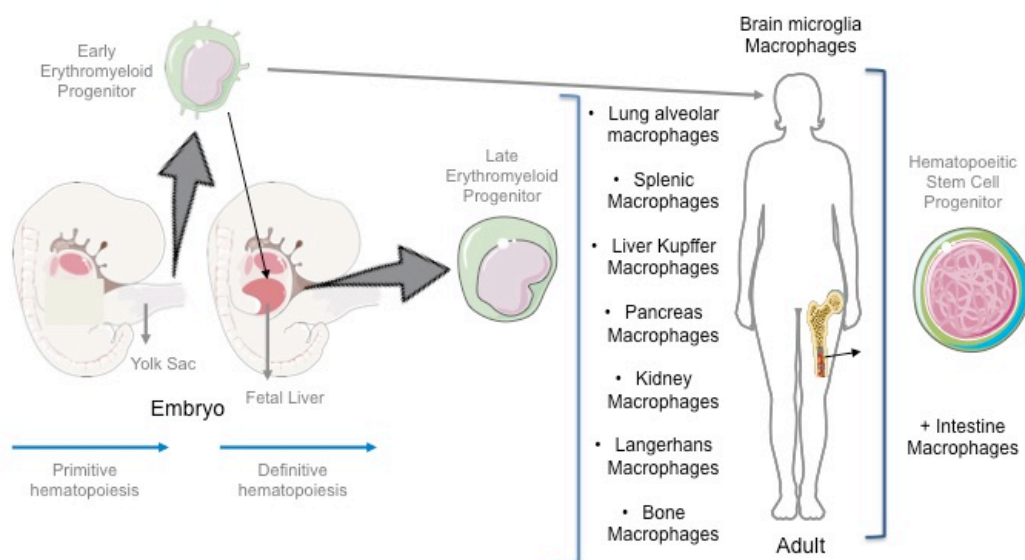


**Figure 3: Macrophages Ontogeny.** Based on Guerriero 2018.

# 1.2.2.1 Tumour-associated macrophages (TAMs)

Macrophages are able to change their physiology in response to epigenetic modifications or different environmental cues, such as cytokine signals (Galli, Borregaard, & Wynn, 2011; Sica & Mantovani, 2012). Mirroring Th1 and Th2 responses, the bioactive state of macrophages is a dynamic process characterized by different populations with distinct functions such as: classically (M1) and alternatively (M2) activated macrophages. M2 macrophages can be further divided into M2a, M2b, M2c and M2d (Gordon, 2003; Mantovani et al., 2004) (Figure 4).

M1-like or classically activated macrophages play central roles in host defense and anti-tumour immunity. This can be explained by the fact that PAMPs and damage-associated molecular patterns (DAMPs) act in synergy with natural killer and T cell-derived IFN-$\gamma$ in order to polarize macrophages via STAT1. This signaling induces a program characterized by the production of reactive oxygen and nitrogen species that increase microbicidal and tumouricidal activities (Mosser & Edwards, 2008; Sindrilaru et al., 2011). Classical M1 cells are high producers of inflammatory cytokines such as IL-12, IL-23, IL1-$\beta$, TNF-$\alpha$ and IL-6 (Sica & Mantovani, 2012). In tumourigenesis, M1 generally act as potent anti-tumour effectors by antagonizing the suppressive activities of pro-tumoural cells, such as M2 or myeloid-derived suppressor cells (MDSCs) (Nardin & Abastado, 2008). However, macrophages gradually lose their anti-tumour properties and acquire a more pro-tumorigenic phenotype during tumour progression (Lança & Silva-santos, 2012).

M2a or alternatively activated macrophages are anti-inflammatory, regulate wound healing, and their polarization is a programmed response by STAT6-activating cytokines IL-4 and IL-13 (Gordon & Taylor, 2005).

IL-1 receptor ligands, immune complexes (ICs) and lipopolysaccharides (LPS) elicit M2b polarization and they are considered immune regulators. They secrete IL-1, IL-6, IL-10 and also TNF-$\alpha$. The third type, M2c, is elicited by IL-10, TGF $-\beta$ and glucocorticoids. Usually they are involved in tissue repair and remodeling. M2d activation is elicited by IL-6 and is known to enhance the induction and growth of tumour cells through angiogenesis (Duluc et al., 2007; Y. Liu & Cao, 2015; Martinez & Gordon, 2014; Mosser & Edwards, 2008).

In general, M2-like macrophages have mainly immunosuppressive activity (Murray & Wynn, 2011) and these pro-tumor macrophages can display pleiotropic functions within tumors, from enhancing tumor cell survival and proliferation (Mantovani, Marchesi, Malesci, & Laghi, 2017), to inducing angiogenesis (Ono, Torisu, Fukushi, Nishie, & Kuwano, 1999), and suppressing local anti-tumor lymphocyte-based immunity (Ruffell et al., 2014; Zelenay et al., 2015). They also promote cancer dissemination through extracellular matrix reorganization (Finkernagel et al., 2016), and consequently both lymph and blood intravazation that promotes metastization (Storr et al., 2012).

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Therefore, macrophages can either maintain tissue homeostasis by serving various housekeeping functions, including host defense, inflammatory responses and anti-tumour capacities or be subverted by continuous insult, suppressing these functions.

*In vitro* and *in vivo* studies have revealed that macrophages can mediate chemotherapy resistance by providing specific survival factors and/or activating anti-apoptotic programs in cancer cells (Castells et al., 2012; Correia & Bissell, 2012). Prognostic significance of TAMs in human cancers has been established by epidemiological evidence showing a positive correlation between high numbers of TAMs and poor patient outcome in some types of cancer, namely head and neck, oral, thyroid, lung, breast, liver, pancreatic, bladder, kidney, ovarian and endometrial cancers as well as Hodgkin lymphoma (Gentles et al., 2015; Ruffell & Coussens, 2015; Steidl et al., 2010; Q. Zhang et al., 2012). Still, there are few cases where high TAM density has also been associated with increase in survival in other types of cancers, such as colon and gastric. This means that the prognostic significance of TAMs is still controversial and highlights the importance of establishing the type/stage of cancer and the phenotype and functional activities of the TAMs (Forssell et al., 2007; Ruffell & Coussens, 2015; Satoshi, O. Hiroyuki, I. Dhar, D. Toshiyuki, F. Shuhei, U. Mitsuo, T. Nobutaka, S. Masaki, I. Gen Ichiro, 2003; Q. Zhang et al., 2012).
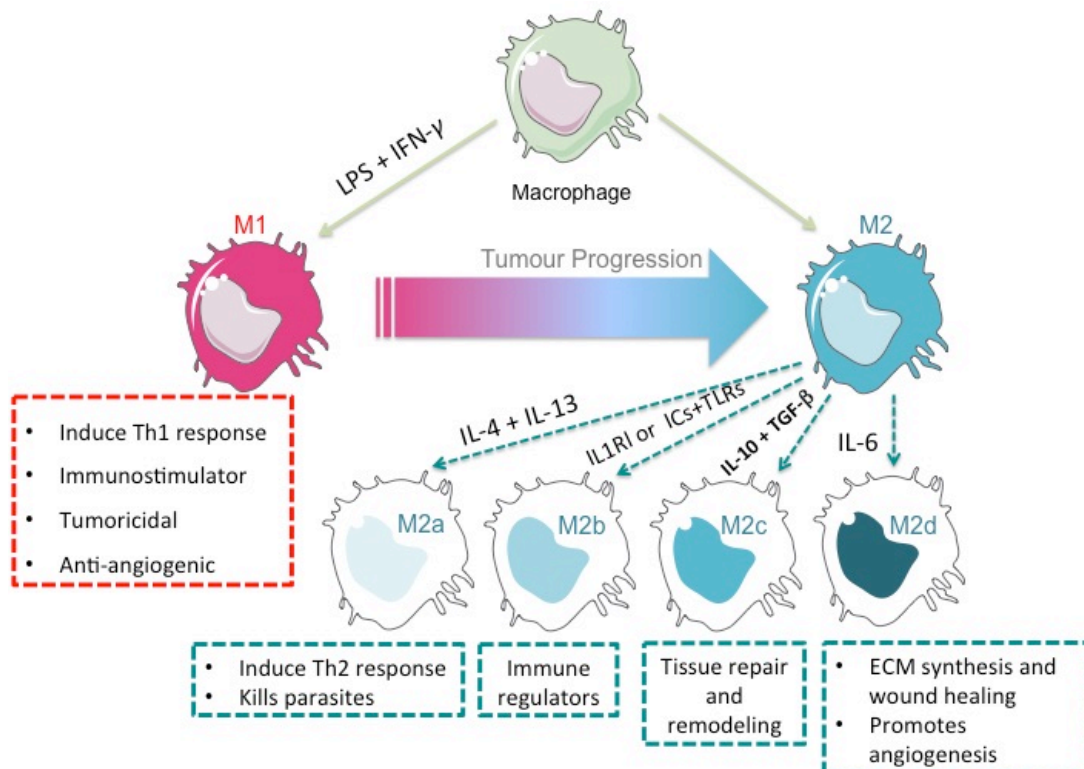


**Figure 4: Macrophage polarization during tumour progression.** Based on Weagel, 2015.

## 1.2.3. Neutrophils

In humans, neutrophils account for 50% to 70% of all circulating leukocytes, and they have a very short half-life of few hours in the circulation. Not surprisingly, more than half of the bone marrow is dedicated to the production of neutrophils (Beutler, 2004; Edwards, 1994). Like other hematopoietic cells, neutrophils are formed in the bone marrow in response to several cytokines, principally to granulocyte colony-stimulating factor (G-CSF) (Borregaard, 2010).

In order to respond to invaders, neutrophils have specific receptors that are able to induce intracellular signals that lead to pathogen-killing capacities. In response to pro-inflammatory stimuli in the tissue, neutrophils rapidly accumulate at sites of infection and inflammation, being specialized at exerting anti-microbial activities. This fully activated state of neutrophils is characterized by the acquisition of phagocytic capabilities, generation of reactive oxygen species (ROS), release of granule proteins and neutrophil extracellular traps (NETs) (Pitrak, 1997; Stuehr & Nathan, 1989).

As a matter of fact, when performing NETosis we can say that they act like "kamikazi". Upon infection, neutrophils follow chemokine gradients in order to target the invader but it simultaneously causes collateral tissue damage through the discharge of toxic mediators that also result in their own death (C. Nathan, 2006).

## 1.2.3.1. Tumour-associated neutrophils (TANs)

Like macrophages, neutrophils are also highly plastic within the tumour microenvironment. Therefore, they can either exert anti-tumour functions (N1) or pro-tumoural functions (N2). Indeed, evidence from animal models suggests that towards distinct tumour-derived signals (e.g. TGF-$\beta$ or the chemokine ligand 17 (CCL17)), neutrophils are able to shift from an anti-tumoral to a more pro-tumoural phenotype (Fridlender et al., 2009; Mantovani, 2009; Mantovani, Cassatella, & Costantini, 2011; Mishalian et al., 2014).

Several epidemiological evidences suggest that neutrophil infiltration within some types of tumours may be associated with poor outcome. Neutrophils have been reported to support tumour growth not only by the production of angiogenic factors and matrix-degrading enzymes (Fridlender et al., 2009; Gregory & Houghton, 2011; Pekarek, Starr, Toledano, & Schreiber, 1995; Shojaei, Singh, Thompson, & Ferrara, 2008), but also through promotion of metastasis (Mantovani et al., 2011; Tazawa et al., 2003) and suppression of anti-tumor immune responses (Mócsai, 2013; Schmielau & Finn, 2001). This negative correlation has been reported in human gliomas (Fossati et al., 1999), metastatic and localized renal cell carcinoma (Donskov, 2013; Jensen et al., 2009), head and neck (Trellakis et al., 2011) esophageal squamous cell (J. Wang

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et al., 2014), bronchoalveolar and hepatocellular carcinomas, and also in pancreatic neoplasias (Reid et al., 2011). Conversely, in few instances, TANs have also been associated with good prognosis, for example in gastric carcinoma (Caruso et al., 2002). Thus, similarly to their myeloid's cousin macrophages, the role of neutrophils within the tumours may be different, depending on several factors (e.g. type of the tumour) and there is still a lot of work to do in order understand if their presence is associated with good or bad prognosis and if this can be associated to a specific type of cancer (Caruso et al., 2002; J. jing Zhao et al., 2012; Q. Zhao et al., 2012).

In mouse models, TAN depletion led to a decrease in tumour growth (Fridlender et al., 2009; Nozawa, Chiu, & Hanahan, 2006; Pekarek et al., 1995). For instance, these cells can become more pro-tumorigenic (N2) in the presence of TGF-$\beta$ and blocking this cytokine converts neutrophils to a more anti-tumorigenic (N1) phenotype, which makes them more capable of killing tumor cells and reduces the level of immunosuppression in the tumor microenvironment (Fridlender et al., 2009). By contrast, IFN-$\beta$ has been suggested to polarize neutrophils to the N1 phenotype (Jablonska, Leschner, Westphal, Lienenklaus, & Weiss, 2010).

## 1.2.4. Myeloid-derived Suppressor Cells

In the last few years, MDSCs have emerged as negative regulators in many pathologic conditions, including cancer. These suppressor cells can be divided in two large groups: granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). While PMN-MDSCs are very similar to neutrophils, M-MDSCs are more identical to monocytes (Gabrilovich, Ostrand-rosenberg, & Bronte, 2012). A third small population of MDSCs currently termed as early-stage MDSCs has also been described and includes cells with colony-forming activity and other myeloid precursors. However they only have been yet described in humans (Dumitru, Moses, Trellakis, Lang, & Brandau, 2012).

MDSCs display a more immature phenotype and morphology, leading to a relatively weak phagocytic activity, high expression of arginase and secretion of anti-inflammatory cytokines (Umansky, Blattner, Gebhardt, & Utikal, 2016; Youn, Collazo, Shalova, Biswas, & Gabrilovich, 2012). This phenotype is strongly associated with functional immunosuppression and therefore, intensive clinical studies already identified MDSCs as a valuable predictive marker in cancer and extensive efforts in MDSC targeting are ongoing (Solito et al., 2014). For instance, recent studies in mice already showed that inhibition of MDSCs during immunotherapy also increases its therapeutic effect (Davis et al., 2017; Du Four et al., 2016; Highfill et al., 2014; Iida et al., 2017; Kamran, Kadiyala, & Castro, 2017; Lu et al., 2017).

Of note in the present work we have refrained to use the nomenclature of MDSC and favoured the usage of myeloid cell lineage associated with their

localization.

## 2. Nature of Cancer

Tumours show marked heterogeneity between tumour types (brain, lungs, kidney), patients with the same tumour and within the tumour mass itself (e.g. clonal evolution) (Weinberg, 2014).

The hallmarks of cancer by Hanahan and Weinberg, recognized ten major characteristics that are: proliferative signaling, evasion from growth suppressors, resisting apoptosis, unlimited multiplication, stimulating angiogenesis, promotion of invasion and metastasis, genome instability and mutation, reprogramming of energy metabolism, evasion from immune destruction and tumour enhanced inflammation (Hanahan & Weinberg, 2011).

Tumours can arise both by internal factors (germline or somatic mutations) and environmental factors (tobacco, diet, radiation, infections) (Weinberg, 2014).

Internal factors, driven by (hereditary/germline or acquired/somatic) genetic alterations have been described. For instance, members of the Ras pathway are well-known oncogenes that are commonly mutated, leading to augmented or constitutive activation, in several types of cancer. Dysregulation on this signaling pathway can give rise to several neoplasias, such as hepatocellular carcinoma (L. Li et al., 2016), melanoma, non-small cell lung carcinoma (NSCLC), ovarian or thyroid cancers (Burotto, Chiou, Lee, & Kohn, 2014). Contrarily, genetic alterations leading to inactivation of the p53 protein, a tumour suppressor gene, also known as the guardian of the genome, is very common in almost all human cancers, such as brain, esophagus, lung, breast, liver, reticuloendothelial and hematopoietic tissues (Hollstein, Sidransky, Vogelstein, & Curtis, 1991).

The environment in which people live contributes a lot to incidence of neoplasms. This major risk factor can be demonstrated by several epidemiological studies of migrant populations, for example. Japanese rates of stomach cancer can be 6 to 8 times higher if we compare with Americans in Hawaii. Nonetheless, when Japanese people migrate into Hawaii, within a generation their cancer rates start to be similar to Hawaii's. This suggests that the differing cancer rates are not due to genetic differences between the Japanese and the Hawaii's people (Peto, 2001). Other example is the Seventh-Day Adventists, whose religion does not support smoking habits, heavy drinking and consumption of meat. What was found was that they die much less from cancer compared to the general population. Hence, all these epidemiological studies indicate that our lifestyle and environment are also one of the dominant determinants of cancer incidence (Anand et al., 2008; Weinberg, 2014).

The link between inflammation and cancer is not new. In 1863, Virchow hypothesized that the origin of neoplasms was at sites of chronic inflammation

by the observation of leukocytes infiltration within tumours. Moreover, many malignancies are indeed initiated by infections. For instance, Hepatitis C in the liver predisposes to liver carcinoma and chronic *Helictobacter pylori* infection is the world's leading cause of gastric cancer (Beaugerie et al., 2013; Ernst & Gold, 2000; Kuper, Adami, & Trichopoulos, 2000; Shacter & Weitzman, 2002). Infections by viral agents may also transform cells by inserting active oncogenes into the host genome. Examples of these are the human papilloma virus (HPV), human herpesvirus 8 (HHV8) or Epstein-Barr virus associated with cervical carcinoma, Kaposi's sarcoma and lymphomas, respectively (Boshart et al., 1984; Mesri et al., 1996; Ok, Papathomas, Medeiros, & Young, 2013). In addition, it is estimated that 15 to 20% of all deaths from cancer worldwide are liked to chronic inflammation that predisposes individuals to several types of cancer (F. Balkwill & Mantovani, 2001). Indeed, clinical evidence supports the strongest association between chronic inflammation and neoplasms in people with inflammatory bowel diseases, such as chronic ulcerative colitis or Crohn's disease, which are more susceptible to get colon cancer.

## 2.1. Tumour microenvironment (TME)

The TME is a complex framework, densely populated by fibroblasts, myofibroblasts, mesenchymal stem cells, adipocytes and extracellular matrix (ECM). It is also made of recruited/expanded cell types, such as growing blood vessels and immune cells, both of lymphoid and myeloid origins (Coussens, Zitvogel, & Palucka, 2013; Hanahan & Weinberg, 2011; Mantovani, Allavena, Sica, & Balkwill, 2008). Collectively, tumour-infiltrating immune cells can shape the disease course, influence response to treatment and consequently have an important prognostic value (Gentles et al., 2015).

Tumour progression results from the crosstalk between these different immune cell types, the surrounding supporting tissue (stroma) and the tumour cells themselves (F. R. Balkwill & Mantovani, 2012; Gordon & Martinez, 2010). Typically, tumour-infiltrating myeloid cells can represent up to or over 50% of the TME and they may exert a dual role on tumour development and progression. Hence, immune cells can directly eliminate nascent tumour cells or be part of an anti-tumoural response, but they can also be recruited and trained by tumour cells to promote tumourigenesis (discussed on section 2.2. Cancer Immunoediting) (Grivennikov, Greten, & Karin, 2011; Hanahan & Weinberg, 2011).

## 2.2. Cancer ImmunoEditing: from immune surveillance to escape

In 1883, William Coley used live bacteria (streptococcal organisms) to treat cancer and at the time, when the immune system was still unknown, he thought that it was the infection itself that was inducing cancer shrinkage. It

took many more years and a better understanding of the lymphocytes to realize that the immune system could recognize transformed malignant cells as non-self and control their growth (Y. Yang, 2015). The evidence that the immune system could in fact control tumour growth led first Lewis Thomas and some years later Frank Burnet to propose the concept of immune surveillance (Swann & Smyth, 2007). This theory defends that cells and tissues are constantly monitored by an ever-alert immune system, and it has the ability to identify and destroy nascent tumours, acting as a primary defense against neoplasms (Hanahan & Weinberg, 2011; Swann & Smyth, 2007). Over the last decade, studies using mouse models and human patients have demonstrated that the immune system continuously patrols tumour formation. For instance, many induced tumours can be rejected by spontaneous immune responses and immunodeficient mice develop higher numbers and faster growing tumours. In addition, tumours formed in normal mice are qualitatively different from those that form in the absence of a functional immune system (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Importantly the presence of tumour-infiltrating lymphocytes (TILs) has been shown to correlate with improved survival of a great variety of solid tumor types, including primary and metastatic melanoma (Clark et al., 1989; Clemente et al., 1996), advanced ovarian adenocarcinoma (L. Zhang et al., 2003), gastric carcinoma (Ishigami et al., 2000), squamous cell lung carcinoma (Villegas et al., 2002) and colorectal cancer (Coca et al., 1997).

Notwithstanding, most recently was demonstrated that the immune system not only can protect the host against tumours but also selects the ones that are less immunogenic, helping the most resistant tumour cells to grow (Dunn, Old, & Schreiber, 2004). This evidence led Gavin Dunn, Robert Schreiber and others to redefine the cancer immunosurveillance hypothesis into cancer immunoediting, which comprises three different phases: *elimination*, *equilibrium* and *escape* (Dunn et al., 2004; Schreiber, Old, & Smyth, 2011) (Figure 5).

The *elimination* phase consists in the recognition and elimination of tumour cells by the immune cells. Molecularly, the role of host effector molecules, such as IFN-γ, perforin, Fas/FasL, NKG2D and TRAIL are well recognized (Dunn et al., 2004).

The following step in cancer immunoediting is the *equilibrium* phase. Some of the sporadic tumour cells that manage to survive immune destruction may then be subjected to immunoediting processes. Thus, this phase involves a continuous elimination of tumour cells at the same time that there is an Darwinian selection of the most resistant and lower immunogenic tumour cells (Dunn et al., 2004). Equilibrium is probably the longest phase and may occur over a period of many years. In fact, it has been estimated that it can be a 20-year interval between initial carcinogen exposure and clinical detection of the tumour (Dunn et al., 2004; Loeb, Loeb, & Anderson, 2003). For instance, the

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appearance of metastatic melanoma ~1-2 years after renal transplantation was observed in two patients from the same donor. Sixteen years before, the same donor was treated for melanoma and was considered tumour-free at the time of the transplantation (MacKie, Reid, & Junor, 2003). We can speculate that cancer cells may have been kept in equilibrium in the donor, but by the continuous administration of immunosuppressive drugs they got activated and started to grow (Dunn et al., 2004).

In the *escape* phase, this new population of tumour clones that is able to sculpt forces of the immune system, can now grow and expand until become clinically detectable (Dunn et al., 2004).

At this stage the inflammatory immune cells and cytokines within the TME can promote, rather than suppress, tumour growth (F. Balkwill & Mantovani, 2001; Mantovani et al., 2008). This dual role by which the immune system can suppress and/or promote cancer growth will be described in more detail below**.**
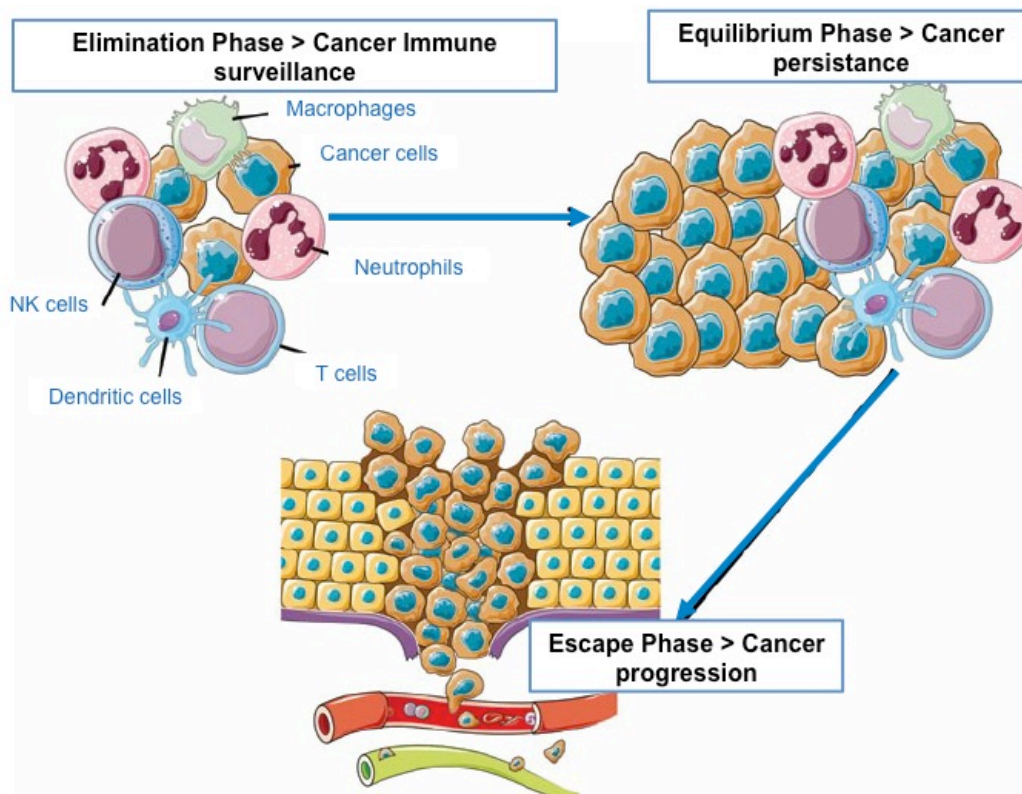


**Figure 5: The three phases of cancer immunoediting: elimination, equilibrium and escape.** Adapted from Garg, et al., 2012.

# 3. Breast Cancer

This project is focused on breast cancer because it is the most commonly diagnosed type of malignancy and the first leading cause of cancer-related death in women (Ferlay et al., 2015). There are several risk factors for breast cancer,

like genetics, age, low rates of breastfeeding, being overweight, lack of exercise, smoking and eating unhealthy food (Singletary, 2003). Breast cancer can be a highly curable disease when detected early, but it can become a mortal disease when discovered too late. Therefore, access to high quality of care leading to early diagnosis can be very important (Barnard, Boeke, & Tamimi, 2015).

The way breast cancer is categorized and treated is largely based on biology-driven therapies. This is a perfect example on how understanding the physiology of the tumour cells led to development of very efficient treatment strategies. Nowadays, the most useful way to predict prognosis and responsiveness to treatments is stratifying patients accordingly to the expression of three receptors on breast cancer cells. Thus, immunohistochemistry (IHC) is widely used to assess the expression of estrogen receptor (ER), progesterone receptor (PR) and overexpression of human epidermal growth factor receptor 2 (HER2+). When none of the three receptors are expressed, this type of breast cancer is defined as triple-negative breast cancer (TNBC). This specific type of neoplasm makes up 10-30% of all breast cancers, is associated with younger age, higher stage at diagnosis and therefore, poorer prognosis. When these patients are treated with neoadjuvant chemotherapy and show a pathological complete response on resection, then prognostic is very good. However, the prognosis is worse and with a higher incidence of recurrences when patients fail to show a pathological complete response (Jia, Shanmugam, Sethi, & Bishayee, 2016; Masuda et al., 2013; Murria et al., 2015). Thus, novel therapeutic strategies are critically needed for relapsing patients that become resistant to chemotherapy.

While the therapeutic potential of biology-driven therapies has been exploited, it is clear that the immunologic response modification has not yet been fully explored in breast cancer. Stunning successes of cancer immunotherapy in melanoma, lung cancer, acute lymphoblastic leukemia and other cancers reflect the power of T cell immunity. Although, for a long time breast cancer was considered "cold" (that is with low immune infiltrate), growing evidence strongly suggest that immune status is a useful marker to predict the risk of primary and recurrent or metastatic (secondary) breast cancer. In fact, T cells and NK cells (and their cytokine release patterns) are implicated in both primary and secondary prevention of breast cancer. This leads to the hypothesis that immunotherapy might be an option for treatment of breast cancer patients. After a lagging start, efforts to develop effective immune therapy for patients with breast cancer are beginning to raise hope. However, a better understanding of the immune responses in breast cancer will be critical to the design of novel strategies. In the next sections, we will discuss the tumour-immune cell infiltrate in relation with prognosis and the different strategies for immunotherapy in general and applicable to breast cancer.

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## 3.1. TAMs in breast cancer

Macrophages are key players in the normal physiology of the breast, and consequently they are also implicated in the pathologic breast cancer conditions (Gouon-Evans, Lin, & Pollard, 2002). Interestingly, TAMs are prominent components of the immune infiltrate in the breast cancer microenvironment and are correlated with poor prognosis in patients with breast cancer (Mantovani, Marchesi, Porta, Sica, & Allavena, 2007; Noy & Pollard, 2014), since their inhibition leads to tumour growth delay. For instance, by using a highly selective small molecule that blocks CSF1R signalling, which drives the recruitment of TAMs to the tumour milieu, the number of macrophages decreases and it is accompanied by an increase in the infiltration of CD8+ T cells and limited mammary tumor growth (Strachan et al., 2013). In addition, by blocking the signalling of IL-10, one of the main by-product of TAMs, it was possible to improve the response to standard chemotherapeutic agents (paclitaxel and carboplatin) by enhancing the expression of IL-12 by intratumoural DCs and promoting a productive CD8+ T cell response (Ruffell et al., 2014).

As tumour-drivers, TAMs can affect the course of breast cancer development in several ways, including tumour progression and therapeutic resistance. It has been already described that TAMs, within tumour bed, may release "chemoprotective" factors, such as cathepsin B and cathepsin S that can protect tumour cells from the cytotoxic effects of several chemotherapeutic agents. In fact, TAMs have been already associated with tamoxifen resistance in postmenopausal patients (Xuan et al., 2014).

TAMs also promote metastasis formation (J. Chen et al., 2011; Quian & Pollard, 2010). For instance, a specific G protein-coupled to receptor 132 (Gpr132) acts as a sensor on macrophages and they can respond to the lactate that it is released by cancer cells due to the low oxygen supply and increased sugar metabolism. Consequently, macrophages change their phenotype to M2, which facilitates metastization of breast cancer cells. As a result, Gpr132 deletion in mouse models reduces M2 macrophages and blocks the appearance of metastasis in the lung. In fact, lower Gpr132 expression in breast cancer patients positively correlates with metastasis-free survival (P. Chen et al., 2016). Kuan and her team also found that tumour-derived IL-1$\alpha$ acting on TAMs was able to induce the expression of the thymic stromal lymphopoietin (TSLP), which is a critical cytokine for tumour survival. Aggressive types of breast cancer can led to the formation of metastasis and, in this case, TSLP blocker reduced the number of metastasis in the lungs (Kuan & Ziegler, 2018).

Thus, all these evidence that the presence of TAMs is associated with poor prognosis in over 80% of breast cancer cases and resistance to therapy, makes TAMs an attractive target (to deplete or manipulate) for therapeutic intervention (Bingle, Brown, & Lewis, 2002).

## 3.2. TANs in breast cancer

Concomitant to previous findings of a higher presence of TAMs and worse prognosis in breast cancer patients, TANs can also help tumours to grow. A study by Soto-Perez-de-Celis and colleagues showed that the presence of TANs is bigger in the most aggressive histologic subtype of breast cancer: TNBC (Soto-Perez-de-Celis, Chavarri-Guerra, Leon-Rodriguez, & Gamboa-Dominguez, 2017).

The production of G-CSF or TGF-$\beta$ can induce neutrophils to exert pro-tumoural functions (N2) and help tumours to grow faster. Additionally, breast cancer cells can also induce N2 to produce Oncostatin, which is an IL-6-like cytokine that then stimulates cancer cells to secrete vascular endothelial growth factor (VEGF), promoting angiogenesis. The lack of IFN-$\beta$ also induce TANs to secrete matrix metalloproteinases (gelatinase B/MMP-9) that destroy the ECM and consequently promote invasion and metastization of tumour cells in other organs (Fridlender et al., 2009; Piccard, Muschel, & Opdenakker, 2012).

Nonetheless, further research into the functional and molecular characterization of TANs in breast cancer, as well as their relationship with response to treatment and prognosis is needed.

## 3.3. Tumour-infiltrating lymphocytes in breast cancer

Low immune recognition through reduced expression of MHC I and/or increased expression of immunosuppressive molecules that result in a decreased lysis of tumour cells by CD8+ cytotoxic T cells (CTLs) are well-documented escape mechanisms of breast tumour cells (Andre et al., 2013; Caras et al., 2004). Thus, the importance of the immune system in breast cancer is starting to be recognized. For example, recent evidence have shown that tumour-infiltrating lymphocytes (TILs) could predict response to therapy (chemotherapy) and improve prognosis both in HER2+ and TNBC (Adams et al., 2014; Dieci et al., 2015; Loi et al., 2013). More specific, regarding HER2+ therapy with trastuzumab, TILs are very important in the efficacy of this type of treatment (Gennari et al., 2004; Salgado et al., 2015). Trastuzumab results in the activation and/or recruitment of several innate immune cells (such as macrophages and NK cells) and increases the susceptibility of tumour cells to antibody-dependent cell-mediated cytotoxic (ADCC) (Arnould et al., 2006). This leads to release of tumour-antigens that can then serve to activate T cells, which will eliminate the tumour.

We believe that the characterization not only of the subtype, but also the immune infiltrate of breast cancer will allow the identification and stratification of patients that may respond better to different available therapies, making TILs within the TME a useful biomarker for clinical practice.

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# 4. Immunotherapy

Over the past decades, our increased knowledge about the immune system and its importance in the destruction of cancer cells has led to the development of new immunotherapeutic strategies that aim to stimulate the immune system to "fight back". These include vaccination strategies, immune checkpoint inhibitors and T cell therapies, which represent a turning point in modern oncology (Vanneman & Dranoff, 2014; Y. Yang, 2015). Yet, despite all the remarkable clinical results, some cancer patients do not respond to these immunotherapies. As previously described, this resistance is likely due to the ability of tumour cells to manipulate their microenvironment, promoting local immunosuppression that allows escape to immune recognition and elimination (Drake, Jaffee, & Pardoll, 2006).

Activating the immune system has also risks. Some patients develop harmful side effects when their immune system attacks healthy cells, such as in a mild way diarrhea because of inflammation in the intestine and some endocrine disorders, some skin rashes, and in more complicated consequences autoimmune diseases, brain hemorrhages. Nevertheless there have been encouraging results from clinical trials to reduce these side effects (Kroschinsky et al., 2017).

The last few years have seen many promising developments in anti-cancer immunotherapies. However, there is still much work to do to help to get new drugs from the bench through clinical trials and then to the bedside.

## 4.1. Vaccination therapies

The introduction of vaccines against infectious agents revolutionized medicine by preventing a lot of diseases. In contrast, cancer vaccines are mostly designed to boost the body's natural defenses to fight cancer by inducing cellular immune responses. Thus, the cancer vaccine leads to activation of T lymphocytes specific for tumour associated-antigens (TAA) or tumour specific-antigens (TSA) that will allow recognition and elimination of tumour cells.

U.S. Food and Drug Administration (FDA) has already approved two types of cancer vaccines: prophylactic vaccines against the hepatitis B virus (HBV) and human papilloma virus (HPV) to prevent liver and cervical cancers, respectively (Ogholikhan & Schwarz, 2016; Stanley, 2007). For treatment, there is one dendritic cell-based vaccine for patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC), called Sipuleucel-T (Provenge) (Cheever & Higano, 2011). This vaccine consists in autologous transplant of peripheral-blood mononuclear cells (PBMCs), including APCs. They are activated *ex vivo* with PA2024, which is a recombinant fusion protein constituted by a prostate antigen (prostatic acid phosphatase - PSA) conjugated with an immune-cell activator (GM-CSF) (Kantoff et al., 2010). After their activation, they are re-injected into the patient and are efficient in the

activation of the immune system against PSA-expressing tumour cells. Sipuleucel-T showed a benefit on OS of patients in three double blind randomized phase III clinical trials: D9901, D9902A and IMPACT.

Several other vaccines that have as their principal target, specific tumour antigens, have also shown promising results, like e75 peptide/NeuVax, which is a peptide vaccine for the treatment of HER2+ breast and also ovarian cancers (Chablani, 2013; Mittendorf, Holmes, Ponniah, & Peoples, 2008). PROSTVAC-VF is being used for the treatment of mCRPC and is a genetic vaccine that combines the PSA and three co-stimulatory molecules for T cell activation (Madan, Arlen, Mohebtash, Hodge, & Gulley, 2009). Other therapeutic cancer vaccine that got surprising results in patients with NSCLC is CIMAvax-EGF, which generates a specific humoral response against the epidermal growth factor (EGF) itself. This vaccine also comprises the *Neisseria meningitis* outer protein P64k and Montanide ISA 51 as adjuvants in order to potentiate the immune response (Saavedra et al., 2018).

Another approach is also to target biologic features that are important for the growth of the tumour, like the blood supply. Thus, in order to avoid the formation of new blood vessels, an anti-angiogenic vaccine from placenta-derived endothelial cell lysates pretreated with IFN-$\gamma$ to enhance immunogenicity, has also being developed: ValloVax (Wagner et al., 2015, 2017).

Although several preclinical studies in mice have provided great evidence of the capacity of this type of vaccines, clinical translation has been proved to be challenging. For instance, critical questions regarding this approach that need to be solved are the development of a new way to overcome the antigen immunogenicity and tumour immunosuppression (Jacques Banchereau & Palucka, 2017; Bowen, Svrivastava, Batra, Barsoumian, & Shirwan, 2018).

## 4.2 Immune checkpoint inhibitors (ICI)

One of the tumour defense mechanisms is to inhibit specific cells of our immune army by interfering with ICI pathways. Hence, tumour cells can by themselves or by promoting the microenvironment induce engagement of two of the very well-known co-inhibitory molecules expressed by T cells, namely cytotoxic T lymphocyte antigen 4 (CTLA-4) and program cell death 1 (PD-1).

CTLA-4 plays a critical role in the priming phase of the immune response and its binding inhibits T cell activation in lymphoid organs, leading to a decrease of the production of IL-2 and arrest of cell cycle (Brunner et al., 1999). Programmed death protein 1 (PD-1) also acts as a negative regulator that limits the production of IFN-$\gamma$ and T cell proliferation, increases T cell apoptosis and contributes to T cell exhaustion in peripheral tissues (Dong et al., 2002). Therefore, to fight cancer, these brakes need to be removed in order to get a stronger immune response. Accordingly, antagonist monoclonal antibodies have been generated.

The monoclonal antibody (mAb) Ipilimumab, also known as Yervoy, is a blocking antibody for CTLA-4 and it got FDA approval in 2011 for patients with unresectable or metastatic melanoma. Patients that received this treatment were followed up for 10 years and showed an overall survival (OS) of 9.5 months with a 3-year survival rate around 21% (Hodi et al., 2010; Schadendorf et al., 2015). Several mAbs against PD-1 have also being developed, like Nivolumab and Pembrolizumab, respectively known as Opdivo and Keytruda, have demonstrated to significantly improve the progression-free survival (5.1 months against 2.2 months) and OS (72.1% against 42.1%) of patients with melanoma (without BRAF mutation) (Robert et al., 2015). Presently, Nivolumab was already approved for several types of neoplasias, such as renal cell carcinoma (RCC), Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), urothelial carcinoma (UC), colorectal cancer, hepatocellular carcinoma (HCC) and colorectal cancer (with high microsatellite instability). Regarding Pembrolizumab approval, it is already used for the treatment of melanoma, NSCLC, HNSCC, Hodgkin lymphoma, UC and gastric cancer (Gong, Chehrazi-Raffle, Reddi, & Salgia, 2018).

Inhibitors of the principal ligands of these receptors, such as programmed death-ligand 1 (PD-L1), are also being used in the clinic, for example Atezolizumab, known as Tecentriq, for NSCLC and Avelumab, known as Bavencio, for UC (Gong et al., 2018).

Combinations blocking these two axis (CTLA-4 and PD-1/PDL-1) leads to an increase of the efficacy of these cancer therapies in comparison with single treatments (Peng, Lizée, & Hwu, 2013; Reck et al., 2013; Wolchok et al., 2013). In addition, several combinations, including the concomitant or sequential evaluation of chemotherapy or radiotherapy with immunotherapy are already providing significant results. Therefore, it is likely that the future of immunotherapy is combining different approaches to create trained anti-tumour lymphocytes able to attack and break down the defenses of tumours by unleashing their own immune defense against cancer (Lazzari et al., 2018; Melero et al., 2015).

New inhibitory pathways and drugs blocking CD233 or lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin-3 (TIM-3), CD272 or B and T cell lymphocyte attenuator (BTLA), V-domain Ig suppressor of T cell activation (VISTA), CD73 or adenosin 2a receptor (A2aR) and CD276 or B7 homolog 3 (B7-H3) are also under investigation (Marin-Acevedo et al., 2018; Melero et al., 2015).

Despite the clear efficacy of immunotherapy, regrettably only a small proportion of patients benefit and are sensitive to ICI treatments. Thus, several challenges, including immune-related adverse effects, intrinsic (e.g. absence of tumour antigen, low or down regulation of MHC, change in the antigen presenting machinery) and microenvironmental extrinsic factors (Tregs, MDSCs,

M2 macrophages and other immune checkpoints) can contribute for this resistance (Jenkins, Barbie, & Flaherty, 2018; Linardou & Gogas, 2016; Zaretsky et al., 2016). In order to overcome these issues, it is absolutely necessary to understand these mechanisms for the design of new and better therapeutic strategies.

## 4.3 Adoptive T cell transfer therapy

The basis of adoptive cell transfer (ACT) consists on the infusion of tumor-specific T cells. Various strategies are envisaged for ACT relying on different sources of anti-tumour T cells, and these include TILs, T cell receptor (TCR)-engineered T cells, and chimeric antigen receptor (CAR) T cells. TILs are extracted from patients, selected and expanded *ex vivo* by their ability to kill tumour cells and the best killers are then re-infused back into patients. Usually, their re-administration is made after lymphodepletion and in combination with immunostimulatory agents (Restifo, Dudley, & Rosenberg, 2012; Vacchelli et al., 2013).

The first clinical trial from Steven Rosenberg, demonstrated that this cell transfer therapy induced a remarkable durable complete response in 22% of patients with metastatic melanoma. In the same study, 95% of these complete responses are ongoing beyond the years (S. A. Rosenberg et al., 2011). Furthermore, Dr. Rosenberg and his team also described a very interesting case report of a woman with chemorefractory hormone receptor-positive metastatic breast cancer. She was treated with TILs that were reactive to 4 out of 62 different mutations, in combination with IL-2 and Pembrolizumab. After 22 months, this woman is cancer-free. These results suggest that this type of approach is very promising and because only depends on the mutations and not the cancer type, it might be further extended to a broader spectrum of neoplasias (Zacharakis et al., 2018).

Genetically modified TCR therapies consist in the transference of specific gene sequences to the T cell to encode new TCR $\alpha$ and $\beta$ chains with different peptide specificity. Several clinical trials already showed the feasibility and the potential of this technique in patients with metastatic melanoma or sarcoma. In these cases, T cells were transduced with a TCR directed against the melanoma antigen recognized by T cells (MART-1), melanoma-associated antigen 3 (MAGE-A3), glycoprotein 100 (gp100) and New York esophageal squamous cell carcinoma (NY-ESO-1) (Johnson et al., 2009; Morgan et al., 2006, 2013; Robbins et al., 2011; Sharpe & Mount, 2015).

Another strategy, CAR T cell approach, has been developed in order to improve the potential of ACT. A promising therapy is the so-called chimeric antigen receptor (CAR). In this technique, cells are removed from the blood of patients and then they need to be armed with tumour-specific receptors by genetic engineering before being re-introduced into the patients (Hinrichs &

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Rosenberg, 2014). CARs are constituted by three different parts (from outside to inside): an extracellular tumour antigen recognition domain of the single-chain fragment variant (scFv) derived from an antibody, a transmembrane domain and an intracellular T cell activation domain (C. A. Ramos & Dotti, 2011).

CAR T cells are marking the beginning of a new era in cancer immunotherapy and the real therapeutic value of these genetically engineered cells is being specially demonstrated in B-cell malignancies. In 2010, a case report has shown great results using CD19 CAR T cells as a treatment for lymphoma patients (Kochenderfer, Wilson, Janik, Dudley, & Stetler-stevenson, 2010). Since then, CARs have been shown impressive clinical outcomes and result of that was the FDA first CAR T cell therapy approval - Kymriah (Tisagenlecleucel) - in the summer of 2017 for kids and young adults with B-cell acute lymphoblastic leukemia (ALL).

However, this type of immunotherapy failed to work so well in solid tumours and so far the results are still modest. The presence of physical barriers, antigen loss in tumour cells, lack of unique antigens and the immunosuppressive TME of solid tumours are currently the biggest challenges to overcome (Kato et al., 2017; Yu et al., 2017). Furthermore, CAR T-cells also attack normal tissues involving the heart, lung, brain and liver and this also needs to be taken into account in order to get the best of this therapy (Xia et al., 2017).

Importantly, for all limitations and challenges of these immunotherapeutic strategies, this is why the combination with myeloid cell targeting might be interesting and there are already some promising results with these approaches (discussed on section 5. Myeloid targeted therapy). Thus, given the multifaceted modulatory nature of myeloid cells, we believe that a wider impact on cancer immunotherapy will require reinforcement on the anti-tumour effector mechanisms within the TME.

## 5. Macrophages-targeted therapy

Nowadays, approved cancer immunotherapy aims at harnessing T cells to fight cancer. However, TAMs can display cytotoxic and potent pro-phagocytic functions as well as promote an anti-tumoural microenvironment making them extremely attractive therapeutic targets. In this section we show how a better understanding of the molecular requirements that induce and maintain anti-tumour function in TAMs can lead to development of novel therapeutic options (Figure 6).

## 5.1. Depletion of TAMs

CSF1 through engagement of its receptor CSF1R delivers a critical signal for the generation of monocyte progenitors, not only in the bone marrow

(hematopoiesis) but also for TAMs within tumours. Thus, this pathway is a target to selectively deplete TAMs and consequently prevent them to display pro-tumoural features. For instance, genetic loss of CSF1 results in a delay on mammary tumour progression, leading to a significantly reduced number of metastasis in lung and also in neuroendocrine tumour models, like pancreatic cancer (Lin et al., 2001; Linde et al., 2018; Zhu et al., 2014). Treatment of PyMT mice with paclitaxel in combination with anti-CSF1R significantly reduced the tumour burden, vessel density and increased cytotoxic T cell infiltration compared with treatment with paclitaxel alone (DeNardo et al., 2011). Moreover, CSF1R inhibitors in combination with anti-PD-1 significantly led to tumour regression in melanoma patients that did not respond to immune checkpoint blockade (Neubert et al., 2018).

Overall, preliminary results suggest that targeting the CSF1/ CSF1R axis is a promising strategy. Therefore, several antibodies and small molecules are being tested in clinical trials in various types of tumours, such as pancreatic, prostate and breast cancer (Casseta & Pollard, 2018).

## 5.2. Limiting monocytes recruitment

Other strategy for targeting TAMs is to block monocyte recruitment to the TME through the monocyte chemoattractant protein-1 (MCP-1) also known as CCL2 - C-C chemokine receptor type 2 (CCR2) axis. CCL2 released by tumour cells, or monocytes/macrophages, is a chemoattractant for cells that express the receptor CCR2, mainly monocytes, and also T and NK cells (Deshmane et al., 2009). Thus, inhibition of CCL2 has shown to be correlated with a reduction in tumour growth and metastasis in different models of lung, breast, liver or prostate cancers (Li, et al., 2013). Hence, new CCL2-blocking agents are being tested in clinical trials, such as Carlumab (CNTO 888). Yet, results of these trials are not completely conclusive. In one hand, in metastatic castration-resistant prostate cancer patients, this mAb although well tolerated, did not showed a complete blocking of the CCL2/CCR2 axis nor anti-tumour activity (Pienta et al., 2013; Brana et al., 2015). In another hand, results from other study showed a transient CCL2 suppression and preliminary anti-tumour activity in patients with advanced solid malignances, such as colorectal, prostate or ovarian cancers (Sandhu et al., 2013). Therefore, the future of blocking the CCL2/CCR2 pathway requires a better understanding of the biology of this chemokine receptor. Notwithstanding, a CCR2 antagonist was able to disrupt the CCL2-CCR2 axis, which lead to reduction in the numbers of TAMs, causing the inhibition of tumour growth and metastization in a mouse model of pancreatic ductal adenocarcinoma (Sanford et al., 2013; Nywening et al., 2016).

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## 5.3. Reprogramming of TAMs

Preclinical data indicate that it is possible to educate TAMs to become more tumouricidal and reject neoplastic cells. For instance, IFN-$\gamma$ is able to switch M2 TAMs to M1 phenotype in human ovarian tumours (Duluc et al., 2009). Moreover, zoledronic acid or CpG oligonucleotide are also potential molecules found to repolarize pro-tumoural into anti-tumoural macrophages in mammary and liver mouse tumours, respectively (Coscia et al., 2010; Huang et al., 2012).

Macrophages are phagocytic and they are able to "eat" tumour cells. However, tumour cells can express CD47, which is a "don't eat me" signal that binds to the signal regulatory protein alpha (SIRP$\alpha$) receptor on macrophages, preventing phagocytosis. Irving Weissman has pioneered the therapeutical potential of the CD47/SIRP$\alpha$ axis, for his demonstration that treatment of human non-Hodgkin's lymphoma (NHL)-engrafted mice with a blocking anti-CD47 antibody reduced tumour burden, while combination treatment with rituximab led to elimination and cure of lymphoma (Chao et al., 2010). Intensive work followed and currently various mAb (Hu5F9-G4, SRF231, ALX148, CC-90002) have been designed to block CD47 and TTI-621 is already in clinical trials. Interestingly, a supra molecule consisting of an inhibitor of the CSF1R and a SIRP$\alpha$-blocking antibody was shown to enhance the polarization from M2 to M1 and significantly improved anti-tumour and anti-metastatic efficacies in melanoma and breast cancer models (Kulkarni et al., 2018). A second "don't eat me" signal, the inhibitory receptor LILRB1 that is expressed by macrophages and suppresses their phagocytic activity, is engaged by MHC I molecules on tumour cells, and could provide another therapeutic avenue (Barkal et al., 2017).

Activation of the NF-κB pathway also plays an important role in polarization of TAMs to an anti-tumour phenotype using for instance TLR agonists or anti-CD40 mAbs. Indeed, these agents are not only being tested in clinical trials but some are already in use in the clinic, either as monotherapy or in combination with conventional therapies (Adams, 2009; Yang & Zhang, 2017).

TLR ligands have powerful immunostimulatory properties by inducing antigen uptake, processing and presentation by DCs, leading to T-cell activation and therefore represent promising immunotherapeutic strategy with highest potential to treat cancer (Adams, 2009; Cheever, 2008). FDA already approved both bacillus Calmette-Guerin (BCG) and imiquimod for clinical use. BCG, which stimulates TLR2, TLR4 and also TLR9, is approved for bladder cancer. The use of this agent is also under clinical evaluation for other types of neoplasias, such as melanoma, acute myelogenous leukemia (AML), colon, urotherial and breast cancers (Dols et al., 2003; Gutterman et al., 1976; Morton et al., 1974; Morton et al., 2007; Powles et al., 1975; Vermorken et al., 1999). Imiquimod, which targets TLR7, is indicated for the treatment of basal cell carcinoma and actinic keratosis (pre-malignant condition). In pre-clinical models, TLR7 ligand also showed an

anti-tumour activity in melanoma and breast cancer skin metastasis (Adams, et al., 2008; Adams et al., 2012; Green et al., 2007; Green et al., 2008; Kobold et al., 2016; Menzies et al., 2017).

Although more studies need to be performed, the use of TLR agonists in the clinic is promising and their synergy with standard therapies has been successfully demonstrated both in murine models and clinical trials. For example, using TLR9 ligand (CpG) as a source of antigen in combination with an anti-OX40 antibody showed that this treatment was able to cure, not only multiple types of transplanted mice, but also a spontaneous model of breast cancer (Sagiv-Barfi et al., 2018). Moreover, a specific TLR9 agonist (IMO-2055) has also been tested in lung or renal cancers, for instance. In a clinical trial, NSCLC patients treated with IMO-2055 in combination with erlotinib and bevacizumab showed potential anti-tumour activity (Smith et al., 2014).

CD40 is also an attractive molecule to target given that it is expressed not only by some lymphoma and solid tumour cells themselves but also by immune cells. For instance, CD40 expression has been identified on a range of tumour cells, comprising almost 100% of B cell neoplasms. It is well described that anti-CD40 antibodies can be used to eradicate lymphomas in experimental murine models and also provides protection against re-challenge through rapid induction of cytotoxic T cells (French et al., 1999; Nowak et al., 2003; Todryk et al., 2001). Dacetuzumab and Lucatumumab, two anti-CD40 agonists with high potential, are being tested for hematological malignancies, such as chronic lymphocytic leukemia (CCL), multiple myeloma (MM) and NHL (Hassan et al., 2014).

The effect on these B cell-related cancer types is multiple: it promotes ADCC and antibody-dependent cell-mediated phagocytosis (ADCP), as well as rendering highly immunogenic tumor cells through an increase of tumor-antigen-load and antigen-presenting properties (Rakhmilevich et al., 2012).

In addition, some other solid malignancies also express CD40, such as melanoma and breast, lung, ovary, renal, and bladder cancers (Cooke et al., 1999; Gallagher et al., 2002; Sabel et al., 2000; Thomas et al., 1996; Vonderheide et al., 2007; Wingett et al., 1998). In some of these neoplasms, engagement of anti-CD40 has been shown to inhibit proliferation, but mainly opsonization of the tumour cells render them susceptible to killing by immune cells (macrophages and NK cells) (Bereznava & Chechun, 2007).

Finally, anti-CD40 can also act directly on immune cells (e.g. macrophages, DCs or B cells) and exert its effects by a wide range of different mechanisms to stimulate anti-tumour immunity, including: upregulation of MHC molecules, production of pro-inflammatory cytokines (e.g. IL-12) or boosting APC function (Remer et al., 2017; Casseta & Pollard, 2018).

Currently, there are two agonistic anti-CD40 being tested in clinical trials for solid tumours: CP-870,893 and RO7009789. Although there are some

common adverse effects, including cytokine release syndrome and alterations in immune cell numbers, the treatment has been well tolerated leading to an anti-tumour activity, either alone or in combination with standard chemotherapy (Beatty et al., 2013; Nowak, et al., 2015; Vonderheide et al., 2007; Vonderheide et al., 2013).

Treatment with anti-CD40 mAb in combination with anti-CSF1R antibodies, which was already described in section 5.1 (Depletion of TAMs), has also shown to reprogram TAMs before their depletion, creating a pro-inflammatory milieu able to elicit myeloid and lymphoid responses. Consequently, this lead to an increase not only on anti-tumour efficacy but also in the survival of preclinical models of colon cancer (Hoves et al., 2018; Perry et al., 2018; Verona et al., 2017). Moreover, it was already shown that anti-CD40 mAb together with conventional chemotherapy plus anti-PD-1 and anti-CTLA-4 was able to overcome the resistance to immune checkpoint blockade in the poorly immunogenic pancreatic cancer (Winograd et al., 2015).

Altogether, preclinical and clinical studies on TAMs have shown encouraging progress and targeting them seems to be a promising strategy for cancer treatment. However, concretization as a solid weaponery in cancer therapy requires a better understanding of how the anti-tumour features are regulated, whether all the TAMs can perform them or there is a labour partition amongst subsets, and whether killing options are selected for specific tumour cell types. Therefore, our project aims to address some of these aspects by using TLR ligands synergizing with anti-CD40 mAbs as potential immunotherapeutic agents.
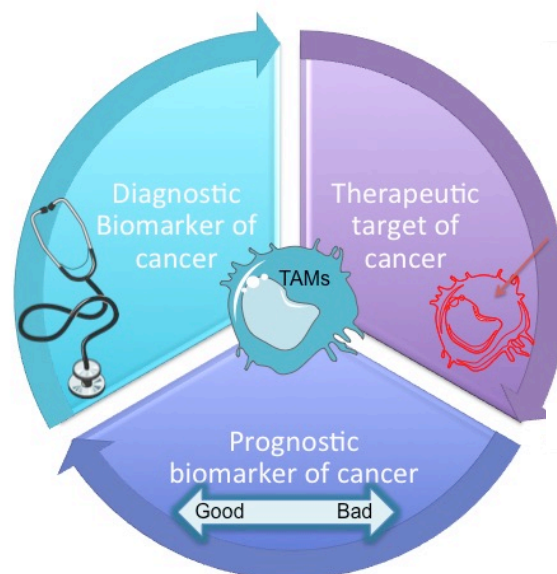


**Figure 6: Clinical application of TAMs.** Based on L. Yang & Zhang, 2017.

# CHAPTER 2: HYPOTHESIS AND OBJECTIVES

Nowadays, there has been a disproportionate attention on pro-tumour myeloid cell functions over anti-tumour myeloid cell functions. We believe that more efforts should be put on understanding how to enhance the protective activity (anti-tumour function) of myeloid cells and in fact, our team has already demonstrated the anti-tumour capabilities of myeloid cells.

One of the papers identified a crosstalk between IL15-dependent patrolling monocytes and NK cells that strongly prevent lung metastasis in primary tumour-bearing mice (Kubo, Mensurado, Gonçalves-Sousa, Serre, & Silva-Santos, 2017). More recently, another study showed that ROS-producing neutrophils could inhibit IL-17+ γδ T cell functions in the tumour bed, suppressing their pro-tumoural functions. All these findings go in accordance with our hypothesis that myeloid cells can have anti-tumour functions and they can crosstalk with different lymphoid subsets (Mensurado et al., 2018).

Our main goal in this thesis was to manipulate myeloid cells (macrophages and neutrophils) and in particular to study their anti-tumour effector functions *in vivo* to eliminate cancer. Thus, we took advantage of their inherent capacity to respond to maturing agents such as TLR ligands and co-stimulatory receptor agonists.

**Building on this, the main objectives of this thesis were:**
- Demonstrate that myeloid cells can be induced to inhibit mammary tumour growth *in vivo*;
- Dissect mechanisms of action of TLR3 ligand with anti-CD40 agonist that act on macrophages and characterize anti-tumour macrophage phenotype and functions;
- Determine the best combination of TLR ligand with anti-CD40 agonist that induces tumour regression in a neutrophil-dependent manner.

# CHAPTER 3: METHODS

## 1. Mice

C57Bl/6J wild-type (WT) mice were purchased from Charles River Laboratories. TCR-$\alpha^{-/-}$, Rag2$^{-/-}$ common gamma chain (Rag2$^{-/-}\gamma$c$^{-/-}$), IFN-$\gamma^{-/-}$ and BATF3 $^{-/-}$ immunodeficent mice were obtained from Jackson Laboratory.

Mice were kept in specific pathogen-free facilities at Instituto de Medicina Molecular | João Lobo Antunes (IMM|JLA) and all experimental procedures were performed according to guidelines approved by the local and national ethics committees.

## 2. Cell lines

E0771 triple negative mammary adenocarcinoma tumour cell line was kindly provided by Dr. Sérgio Dias (IMM|JLA) and the L929 cell line was a kind gift from Dr. Ângelo Chora (IMM|JLA).

Cells were defrosted and maintained in Dulbelcco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS; Gibco by Life Technologies) and 1% of penicillin/streptomycin (P/S; Gibco by Life Technologies) at 37ºC and 5% C0$_2$.

Cells were split every three or four days. For long-term storage, cells were ressuspended in FCS supplemented with 10% dimethyl sulfoxide (DMSO; Sigma) and stored at -80ºC.

## 3. *In vivo* tumour transplantation

During the exponential growth phase, E0771 tumour cells were washed with phosphate buffer saline (PBS), incubated with Tryple Express (Gibco by Life Technologies) for three to four minutes at 37ºC and ressuspended in PBS. One million viable E0771 tumour cells were injected in the mammary fat pad of each mouse in a final volume of $50\mu$l (diluted in PBS).

Tumour growth was measured using a caliper and calculated as (length x width x width)/2 (mm³).

## 4. *In vivo* treatments

To induce tumour regression, treatments were injected directly within the tumour (IT) in a final volume of $50\mu$l. TLR ligands were mixed with $15\mu$g of an agonist anti-CD40 mAb (3.84mg/ml; BioXcell; FGK4.5). We used the following TLR ligands: TLR2/1 (Pam3CSK4) (1mg; Invivogen; tlrl-pms), TLR2/6 (Pam2CSK4) (20 $\mu$g; Invivogen; tlrl-pm25-1), TLR3 ligand (Poly I:C) (50ug; InvivoGen; tlrl-picw), TLR4 ligand (LPS B5 Ultrapure) (2-25ug; InvivoGen; tlrl-

pb5lps), TLR7 (Imiquimod Vaccigrade) (20 $\mu$g; InvivoGen; vac-imq) and TLR9 (CpG) (20 $\mu$g; InvivoGen; tlrl-2395-1).

# 5. *In vivo* depletion and neutralization of specific cell lineages

For macrophage depletion: three and one days before and one day after the treatment followed by every 3 days, clodronate-containing liposomes (Liposoma) were injected as follows 180 $\mu$g intravenous (IV) and 40 $\mu$g intratumoural (IT).

For inducible nitric oxide synthase (iNOS) inhibition: 1000 $\mu$g of Aminoguanidine Hydrochloride (Abcam) or 280 $\mu$g of 1400W (Abcam) were injected intraperitoneal (IP) every day. 50 $\mu$g of Aminoguanine Hydrochloride and 30 $\mu$g of 1400W inhibitions were injected IT every day.

For TNF-$\alpha$ and IL1$\beta$ neutralization: one day before and one day after the treatment, followed by every three to four days, 250 $\mu$g of TNF-$\alpha$ (XT3.11; BioXcell) and 50 $\mu$g of IL1$\beta$ (B122; BioXcell) blocking antibodies were both injected IP. 30 $\mu$g of TNF-$\alpha$ and 20 $\mu$g of IL1$\beta$ were injected IT.

For CD8+ T cell depletion: 200 $\mu$g of anti-CD8 (YTS 156 and YTS 169) were a kind gift from Dr. Luís Graça. Anti-CD8 was injected IP and 50 $\mu$g IT every five days.

For neutrophil depletion: 150 $\mu$g of anti-Gr1 (BioXcell) was injected IV and 50 $\mu$g were injected IT every three and one day before and also every three days after treatment.

# 6. Flow Cytometry analysis

To assess immune response and anti-tumour effector functions, mice were sacrificed and tumours were resected and weighed. Spleen was used for single colour staining.

Tumours were cut into small pieces and digested for thirty minutes at 37ºC with a shaker at 1000 rotations per minute (rpm) in 1.5ml DMEM plus collagenase I (0.4mg/ml; Worthington Biochemical Corporation), collagenase IV (1mg/ml; Worthington Biochemical Corporation) and DNAse (10$\mu$g/ml; Sigma) per tumour. In order to lyse erythrocytes, 500$\mu$l of red blood cell lysis (RBC lysis) (BD Pharma Lyse) was added after tumour digestion to osmotically lyse them.

To assess myeloid or lymphoid cell depletion, blood samples were collected from mice facial vein (~4 drops) into eppendorf tubes with 50$\mu$l of heparin. 500$\mu$l of RBC lysis (BD Pharma Lyse) was added to each blood sample, eppendorfs' were centrifuge for five minutes at 2000 rotations per minute (rpm) and the supernatant was carefully removed. This process was repeated two more times to remove the majority of red blood cells.

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For surface staining, cells were incubated with $25\mu$l of RPMI-1640 medium (cRPMI; Gibco by Life Technologies) supplemented with 10% Fetal Calf Serum (FCS) (Gibco by Life Technologies), 1% penicilin/streptomicin (P/S) (Gibco by Life Technologies), minimum essential medium non-essential amino acids (MEM NEAA) (100X; Gibco by Life Technologies), hepes buffer solution (1M; Gibco by Life Technologies) and sodium pyruvate (100mM; 100X; Gibco by Life Technologies) and 0.1% gentamycin (50ug/ml; Gibco by life Technologies) and 2-mercaptoethanol (50mM; Gibco by Life Technologies). All these in the presence of Fc-block anti-mouse CD16/32 (clone: 93; Invitrogen) with the antibodies presented in Table 1, for one hour at room temperature (RT) and in the dark.

After surface staining, cells were treated with a special dye that allows selective exclusion of dead cells. Various kits were used accordingly to manufacturer's instructions, such as: Zombie Aqua fixable viability kit (Biolegend), Zombie Violet fixable viability kit (Biolegend) and Live Dead fixable Near-IR dead cell stain kit (Invitrogen) for twenty minutes at RT in the dark.

For myeloid intracellular cytokine staining, cells were stimulated with TLR4 ligand (LPS B5 Ultrapure) ($1\mu$g/ml; InvivoGen; tlrl-pb5lps) and IFN-$\gamma$ (50ng/ml; PeproTech) for four hours at 37ºC. For T cell intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (200ng/ml; Sigma) and Ionomycin ($1\mu$g; Sigma) for four hours at 37ºC. Brefeldin-A ($10\mu$g/ml; Sigma) plus Monensin ($5\mu$M; eBioscience) were added during the last two hours. Cells were stained for surface markers and intracellular staining was performed with the Foxp3/Transcription Factor Staining Buffer set (Invitrogen by Thermo Fisher Scientific), following the manufacturer's instructions. Intracellular staining was performed for one hour at room temperature and in the dark or overnight (ON) at 4ºC with monoclonal antibodies (see Table 1).

Cells were analyzed on FACS Fortessa (BD Bioscience). Compensation was performed using cells from the samples analyzed and stained with single antibodies. Data was analyzed using FACS Diva and FowJo software (FlowJo, LLC). Cell gating strategy was performed as described in Figure 1A-B on supplementary data.

*Table 1* - List of antibody clones and respective manufacturer used in flow cytometer analysis. All antibodies are anti-mouse.

| Antibody | Clone | Company |
|----------|-------|---------|
| CD45 | 30F11 | Biolegend |
| CD11b | M1/70 | Biolegend |
| Ly6G | 1A8 | Biolegend |
| Ly6C | HK1.4 | eBioscience |
| F4/80 | BM8 | eBioscience |
| CD11c | N418 | Biolegend |
| MHC II | M5/114.15.2 | Biolegend |
| PD-L1 | 10F.9G2 | Biolegend |
| CD19 | 6D5 | Biolegend |
| CD3 | 17A2 | Biolegend |
| CD4 | GK1.5 | Biolegend |
| CD8 | 53-6.7 | Biolegend |
| IL1β | NJTEN3 | Invitrogen |
| TNF-α | MP6-XT22 | eBioscience |
| iNOS | CXNFT | eBioscience |
| IFN-γ | XMG1.2 | Biolegend |

# 7. *In vitro* tumour cell proliferation assay

In order to assess the effect of TLR ligands and anti-CD40 mAb, E0771 tumour cells were incubated with cell track violet (CTV) (2 $\mu$g/ml diluted in PBS; Molecular Probes) at RT for twenty minutes and in the dark.

After incubation, cells were centrifuged at 1000 rpm for five minutes and washed with cDMEM. Tumour cells were ressuspended in cDMEM and fifty thousand cells were distributed per well in a 6 well plate and incubated with IFN-$\gamma$ (20ng/ml, PetroTech), TLR ligands alone (10ng/ml; InvivoGen) and TLR ligand plus anti-CD40 mAb (6ng/ml; BioXcell) during seventy-two hours. Three days later, cells were washed with 500ul of PBS and incubated with 100$\mu$l of Tryple Express for three to four minutes at 37ºC. cDMEM was added to stop the action of trypsin and cells were transferred to a 96-well plate (V bottom). After centrifugation at 1000 rpm for 5 minutes, supernatant was discarded and cells were also stained to check the expression of MHC I H-2K$^d$ (AF6-88.5; Biolegend), MHC II (M5/114.15.2; Biolegend), as well as the immunosuppressive markers PD-L1 (10F.9G2; Bioloegend) and PD-L2 (TY25, Biolegend). After one hour at room temperature, cells were centrifuge at 1000 rpm for five minutes, the supernatant was discarded and cells were also stained to check cell death with Live Dead (Invitrogen) and Annexin V (Biolegend).

Finally, all the data was acquired in a FACS Fortessa and analyzed using FACS Diva and FlowJo software (FlowJo, LLC).

## 8. *In vitro* bone marrow-derived macrophages (BMDM)

To generate BMDM, bone marrow cell suspension from femurs and tibias of mice were seeded at four million cells in bacterial dishes. These cells were incubated with 10ml of RPMI (Gibco by Life Technologies) supplemented with 10% FCS, 1/5 penicillin-streptomycin and 30% L929 cell supernatant (a mouse fibroblast cell line that produces macrophage colony-stimulating factor (M-CSF)). Three days later, 10ml of RPMI supplemented media was added to the bacterial dishes. On day 6, the supernatant was removed from the culture and attached cells were washed and removed with a pipette. Cells were counted and re-plated in RPMI supplemented media at a density of two million five thousand cells/well (6-well plate). In the next day, cells were polarized with various conditioning media as follows: M0 condition (received media alone containing M-CSF); classical activation M1 condition (received LPS B5 Ultrapure at 0.1 $\mu$g/ml, from InvivoGen; tlrl-pb5lps + IFN-$\gamma$ at 0.05 $\mu$g/ml, from PetroTech); alternative activation M2a condition (received IL-4 at 0.05 $\mu$g/ml + IL-13 at 0.05 $\mu$g/ml, both from PeproTech); M2c condition (received IL-10 at 0.05 $\mu$g/ml, from PeproTech); and stimulation with TLR3 ligand (100$\mu$g/ml; InvivoGen) plus anti-CD40 mAb (5$\mu$g/ml; BioXcell - FGK4.5).

Cells were harvested twenty-four hours post-stimulation and collected either for killing assays, real-time PCR or flow cytometry analysis.

## 9. Killing assay

Twenty thousand E0771 tumour cells were seeded in a 96-well plate (U bottom) and left at 37ºC during, at least one hour, to settle down. One hundred thousand polarized macrophages (after twenty-four hours) were added to the tumour cells. The assay was performed during twenty-four hours in the incubator at 37ºC.

In the next day, Brefeldin-A (10$\mu$g/ml; Sigma) plus Monensin (5$\mu$M; eBioscience) were added during 2 hours and then the cells were stained and analyzed as previously described on section 6. Flow Cytometry Analysis.

## 10. RNA isolation, complementary DNA (cDNA) production, and real-time PCR

Total RNA was extracted using the High Pure RNA Isolation kit (Roche) according to the manufacturer's instructions. mRNA was prepared from BMDM using High Pure RNA Isolation kit (Roche). Reverse transcription was performed with random oligonucleotides (Invitrogen) using MMLV reverse transcriptase (Promega) for one hour at 42ºC. Relative quantification of specific cDNA species to endogenous references *β 2 microglobulin* was carried out using SYBR chemistry on ViiA7 cycler (Applied Biosystems). The $C_T$ for the target gene was

subtracted from the $C_T$ for endogenous references and the relative amount was calculated as $2^{-deltaCT}$. Primer sequences are listed in Table 2.

*Table 2* - List of primers used for Real-Time PCR.

| Primer | Forward | Reverse |
|---|---|---|
| *β2 microglobulin* | 5' - CATACGCCTGCAGAGTTAAGCA | 5' - ATCACATGTCTCGATCCCAGTAGA |
| *Il_1β* | 5' - CGGACCCCAAAAGATGAAGG | 5' - GCCACAGCTTCTCCACAGCCA |
| *Tnf_α* | 5' - TCTTCTCATTCCTGCTTGTGG | 5' - GGTCTGGGCCATAGAACTGA |
| *Nos2* | 5' - CCCTTCAATGGTTGGTACATGG | 5' - ACATTGATCTCCGTGACAGCC |

# 11. Immunohistochemical assays

Mice were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. The tumours were harvested, fixed in formol and processed for paraffin embedding. Serial $4\,\mu$ m sections were used in order to do immunohistochemistry for F4/80 (macrophages), myeloperoxidase (neutrophils), CD3 (T cells) and Von Willebrand Factor (endothelial cells). Briefly, using standard protocols, antigen heat-retrieval was performed in Dako PT module, followed by incubation with the primary and secondary antibodies conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP) for one hour, at RT and in the dark, as shown in Table 2. Tissues were washed and incubated with ENVISION kit (Peroxidase/DAB detection system, Dako Corp) and HIGHDEF red IHC chromogen (AP; Enzo Life Sciences) during thirty minutes, at RT and in the dark. Negative controls included the absence of primary antibodies. Images were acquired in a NanoZoomer (Hamamatsu) and analyzed using Nanozoomer Digital Pathology (Hamamatsu) and ImageJ software (NIH).

*Table 3* - List of primary and secondary antibodies used and respective company.

| Primary antibody | Company | Secondary antibody | Company |
|---|---|---|---|
| FITC rat anti-mouse F4/80 | Biolegend | Peroxidase-conjugated AffiPure Goat anti-rat IgG (H+L) | Jackson ImmunoResearch LABORATORIES, INC. |
| Myeloperoxidase (MPO) rabbit anti-rat | Dako | Polyview Plus AP (anti-rabbit) | Enzo Life Sciences |
| Rabbit anti-human CD3 | Dako | Polyview Plus AP (anti-rabbit) | Enzo Life Sciences |
| Rabbit anti-human Von Willebrand Factor | Dako | Polyview Plus AP (anti-rabbit) | Enzo Life Sciences |

## 12. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc.).

In tumour growth measurement graphs presented in this study, each individual value is plotted.

In other graphs presented, each individual value is plotted and the non-parametric Mann-Whitney test was performed to assess differences between two groups. Every time we had more than two groups to compare, the ordinary one-way ANOVA, followed by Tukey's multiple compared test was performed. Results are presented as p-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and were considered to be statistical significant. Every time we do not present the p-value it means that results are not significant.

# CHAPTER 4: RESULTS

## 1. Effect of TLR ligands in combination with anti-CD40 mAb on E0771 breast tumour cells

Evidence shows that TLRs are expressed not only in immune cells but also in tumor cells, and that the activation of TLRs contributes to both inhibition and promotion of various tumors, with unclear underlying mechanisms (Du, Jiang, Cleveland, Liu, & Zhang, 2016; Shi, Chen, Ye, Yao, & Li, 2016). Thus, we first assessed the effect of various TLR ligands with anti-CD40 mAb in order to assess if this combination could have a direct impact on tumour cells. To address this question, E0771 cells were stained with a cell tracker that allows monitoring their proliferation. TLR ligands plus anti-CD40 mAb, or IFN-$\gamma$ as a positive control (Y. Zhao et al., 2013) to block proliferation, were added and cells were incubated for three days.

Surprisingly, flow cytometry analysis indicated that IFN-$\gamma$ did not affect the proliferation of E0771 cells. The majority of TLR ligands tested *in vitro* had no impact on E0771 cell proliferation. Only TLR7 ligand resulted in inhibition of proliferation (Figure 7A).

Moreover, we also assessed the phenotype of these cells, namely the expression of MHC I and MHC II, which are very important molecules rendering the tumour cells susceptible to an immune response. PD-L1 and PD-L2 expression was also assessed in order to understand if any of the treatments could upregulate these well-established immunosuppressive markers (Figure 7B).

As expected IFN-$\gamma$ induces expression of MHC I and MHC II as well as PD-L1 and PD-L2. TLR2/1 and TLR7 ligand in combination with anti-CD40 mAb showed some differences in the expression of MHC molecules and ligands of PD-1 and also in tumour cell proliferation, respectively (Supplementary figure 2A-E). Thus, we decided to assess the effect of injection of TLR2/6, TLR3, TLR4 or TLR9 ligands in combination with plus anti-CD40 mAb *in vivo*.
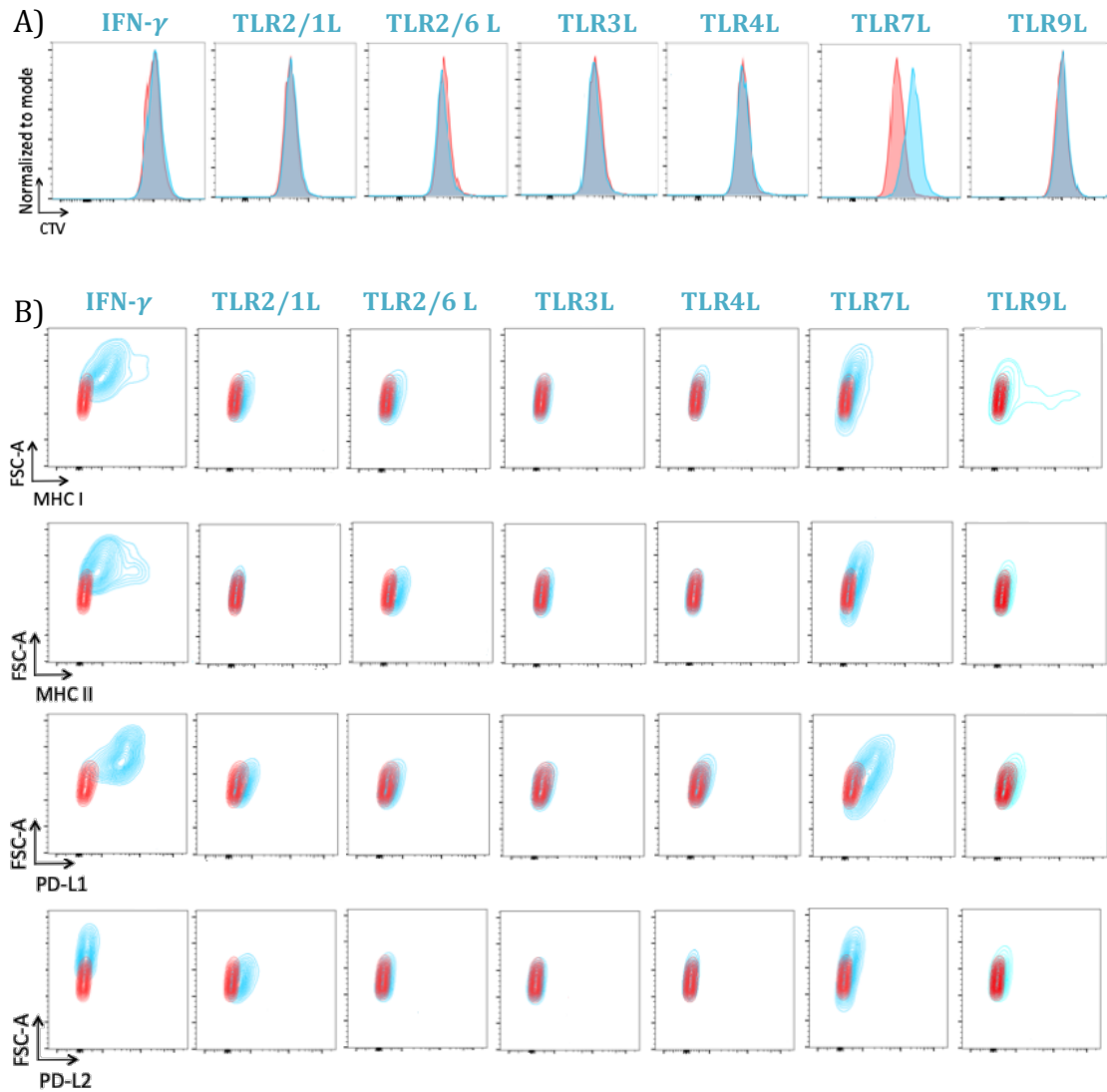
**Figure 7:** *In vitro* **proliferation and phenotype of E0771 tumour cells in the presence of TLR ligands plus anti-CD40 mAb.** Fifty thousand tumour cells were incubated alone (red), with with IFN-$\gamma$ (blue) or different TLR ligands plus anti-CD40 mAb (blue), as indicated in a 6-well plate. Then, seventy-two hours later cells were detached and analysed by FACS. **(A)** Representative histograms of E0771 tumour cells proliferation. **(B)** Representative plots of the expression of MHC I, MHC II, PD-L1 and PD-L2, respectively.

# 2. Effect of intra-tumour injection of TLR ligands plus anti-CD40 mAb on tumour growth *in vivo*

For *in vivo* experiments, mice were transplanted with 1x10$^6$ E0771 breast adenocarcinoma tumour cells in the mammary fat pad.

Tumour growth was monitored and when size reached between 50-100mm$^3$, mice were treated with an intra-tumour injection of individual TLR ligand in the presence of agonist anti-CD40 mAb (Figure 8A).

Intra-tumour injection of all TLR ligands plus anti-CD40 mAb consistently led to a complete remission in most treated animals (Figures 8B-E).
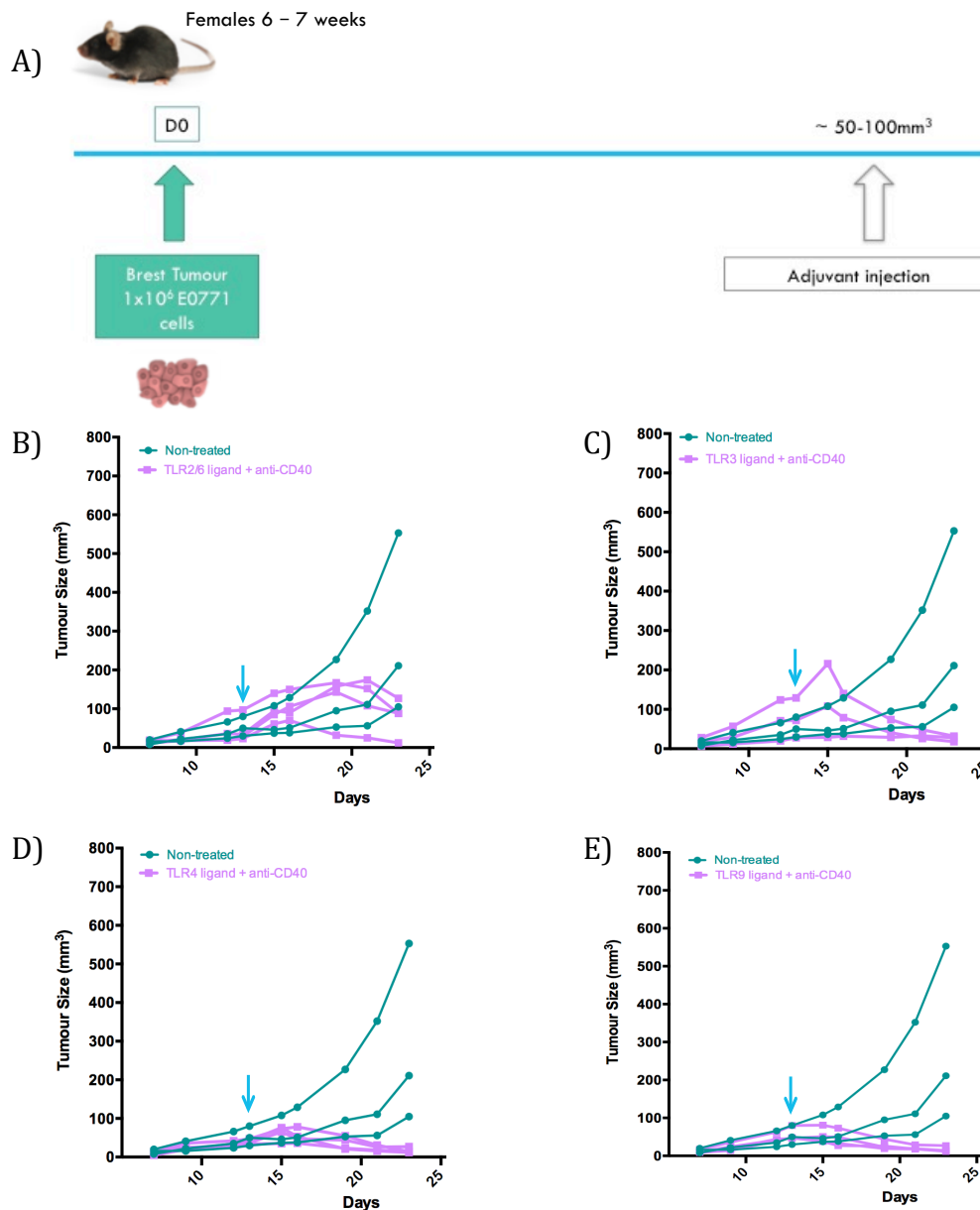
**Figure 8: TLR2/1, TLR2/6, TLR3, TLR4 and TLR9 ligands in combination with anti-CD40 mAb intra-tumour injection induced tumour remission. (A)** Schematic representation of the schedule for tumour injection (green) and adjuvant injection (grey line). E0771 tumour growth in non-treated (blue circle; n=3) and treated mice (purple squares; n=3/4). On day 13 (blue arrow), mice were injected with **(B)** TLR2/6 ligand**, (C)** TLR3 ligand, **(D)** TLR4 ligand and **(E)** TLR9 ligand, all in the presence of anti-CD40 mAb (blue arrow). Statistical analysis was performed using the non-parametric Mann-Whitney test.

TLR3 ligand is a double stranded RNA molecule and it was already reported that viral infections can induce acute inflammation and potent anti-tumour immune responses (Shime et al., 2012). We also confirmed that TLR3 ligand or anti-CD40 mAb alone were not sufficient, and that only the combination led to tumour complete remission *in vivo* (Supplementary figure 3). Besides this, we also concluded this treatment had no direct impact on tumour cell proliferation or changes on the expression of specific markers, as previously shown. Therefore, we decided to dissect further the specific mechanisms of

action by which IT treatment with TLR3 ligand plus anti-CD40 mAb, henceforth called TLR3/CD40 treatment, led to complete remission.

## 3. *In vivo* TLR3/CD40 effect on myeloid lineage

In order to understand if the treatment with TLR3/CD40 was dependent on the myeloid lineage, we used Rag2$^{-/-}\gamma$c$^{-/-}$ mice, which lack B, T, NK and innate lymphoid cell (ILC) function. As Rag2$^{-/-}\gamma$c$^{-/-}$ mice have myeloid cells but lack the lymphoid lineage it is an interesting model to study the importance of each lineage for the treatment outcome. If myeloid cells were irrelevant for the treatment outcome one would expect no differences in tumour growth upon treatment. Interestingly, we found that TLR3/CD40 treatment promoted a delay in tumour growth (Figure 9A-B), which could only be detected by looking to fold increase (Figure 9C). Similar results were observed in TCR$\alpha^{-/-}$ mice which do not have $\alpha\beta$ TCR (CD4+ neither CD8+ T cells) (Figure 9D-E). This suggests that the anti-tumour effect of TLR3/CD40 depends on myeloid cells. However, it also suggests that the complete eradication of the tumour is T-cell dependent.
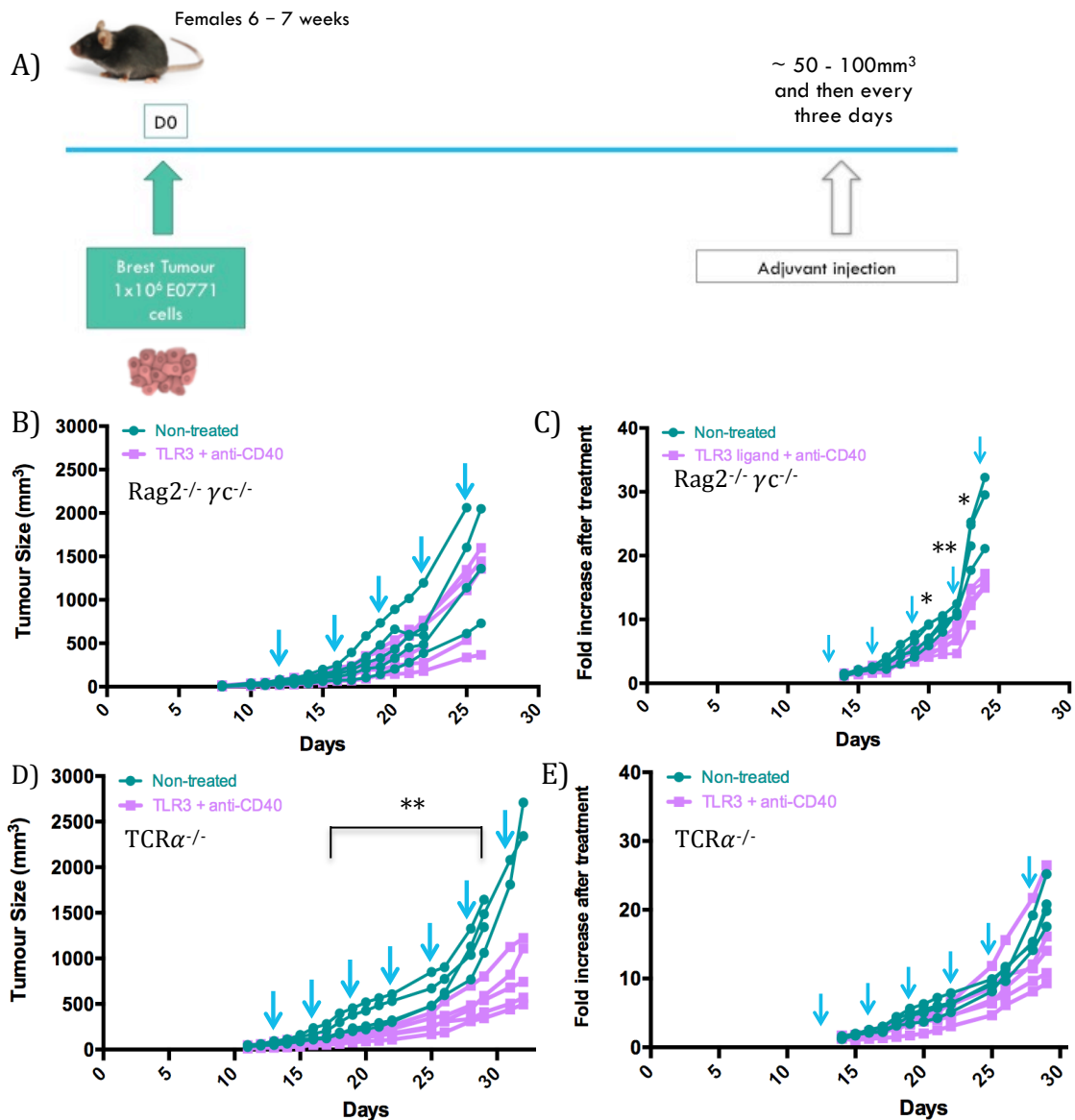
**Figure 9: Tumour growth delay in Rag2$^{-/-}$ $\gamma$ c$^{-/-}$ and TCR $\alpha$ $^{-/-}$ mice. (A)** Schematic representation of the schedule for tumour injection (green) and adjuvant injection (grey line). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=5) and treated mice with TLR3/CD40 IT (purple squares; n=6) every three days since day 13 (blue arrows). **(C)** Fold increase after TLR3/CD40 treatment in non-treated mice (blue circle; n=5) and treated mice (purple squares; n=6) every three days since day 13 (blue arrows). **(D)** E0771 tumour growth in non-treated mice (blue circle; n=4) and treated mice with TLR3/CD40 IT (purple squares; n=5) every three days since day 13 (blue arrows). **(E)** Fold increase after TLR3/CD40 treatment in non-treated mice (blue circle; n=4) and treated mice (purple squares; n=5) every three days since day 13 (blue arrows). Statistical analysis was performed using the non-parametric Mann-Whitney test.

# 4. *In vivo* TLR3/CD40 dependency on macrophages

Macrophages are known to have a dual role during cancer progression (Biswas & Mantovani, 2010; Mantovani, Sozzani, Locati, Allavena, & Sica, 2002; Mosser & Edwards, 2008; Ruffell, Brian; Affara, Nesrine & Coussens, 2012). Therefore, to confirm that these cells played a role in tumour rejection, macrophages were depleted from WT mice using clodronate-containing

liposomes. Consistent with the results already shown by Miguel Pinto in his master thesis (Pinto, 2017), we found that TLR3/CD40 failed to induce tumour regression when macrophages were depleted (Figures 10A-B). Thus, *in vivo* TLR3/CD40 anti-tumour effect depends on macrophages.
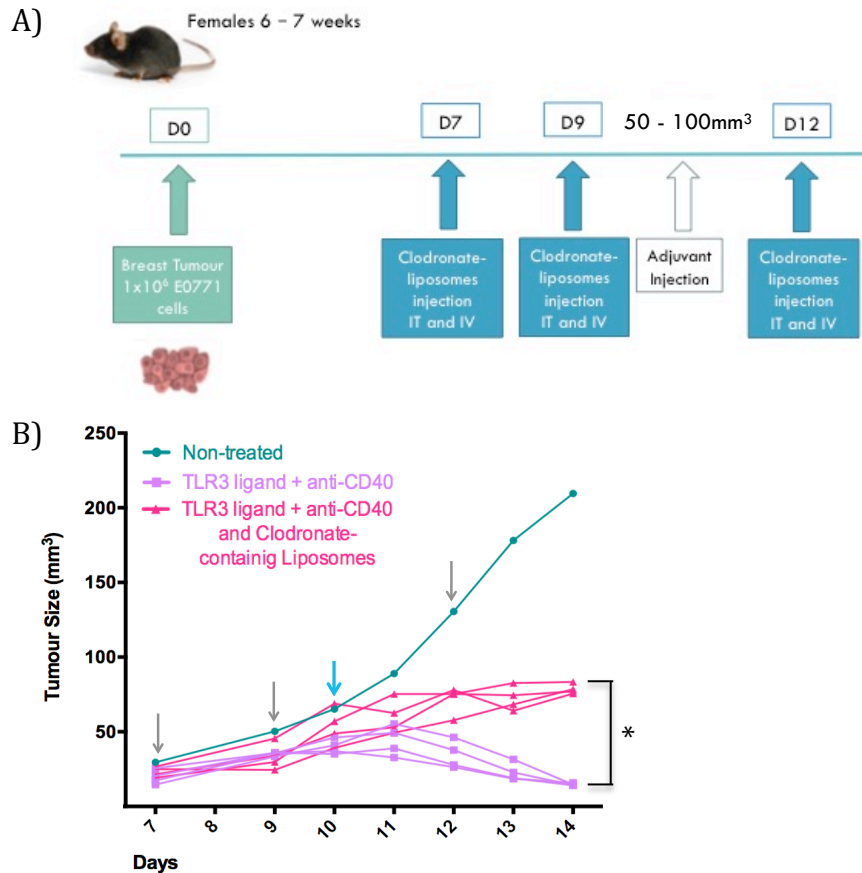


**Figure 10: TLR3/CD40 intra-tumour injection is macrophage dependent. (A)** Schematic representation of the schedule for tumour injection (green), clodronate-liposomes injection both intra-tumour and intravenously every 2 to 3 days (blue) and adjuvant injection (grey line). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=1), treated mice with TLR3/CD40 (purple squares; n=4) and treated mice with TLR3/CD40 and depleted in macrophages with clodronate-containing liposomes (pink triangles; n=4). On days 7, 9 and 12 (grey arrows) mice were depleted in macrophages, both IT and IV and on day 10 (blue arrows), mice were injected with TLR3/CD40 treatment. Statistical analysis was performed using the non-parametric Mann-Whitney test.

# 5.  TLR3/CD40 treatment induces anti-tumour macrophages

Given that the treatment with TLR3/CD40 was dependent on macrophages, the phenotype of these cells was assessed.

Three days after treatment, anti-tumour effectors amongst CD11b$^+$ Ly6C$^{(+/-)}$F4/80$^+$ macrophages were induced. This was revealed by a significant increase in the proportion of TNF-$\alpha^+$ IL-1$\beta^+$ producing macrophages (Figure 11A). We also observed that, in CD11b$^+$Ly6C$^-$F4/80$^+$ macrophages, there was an

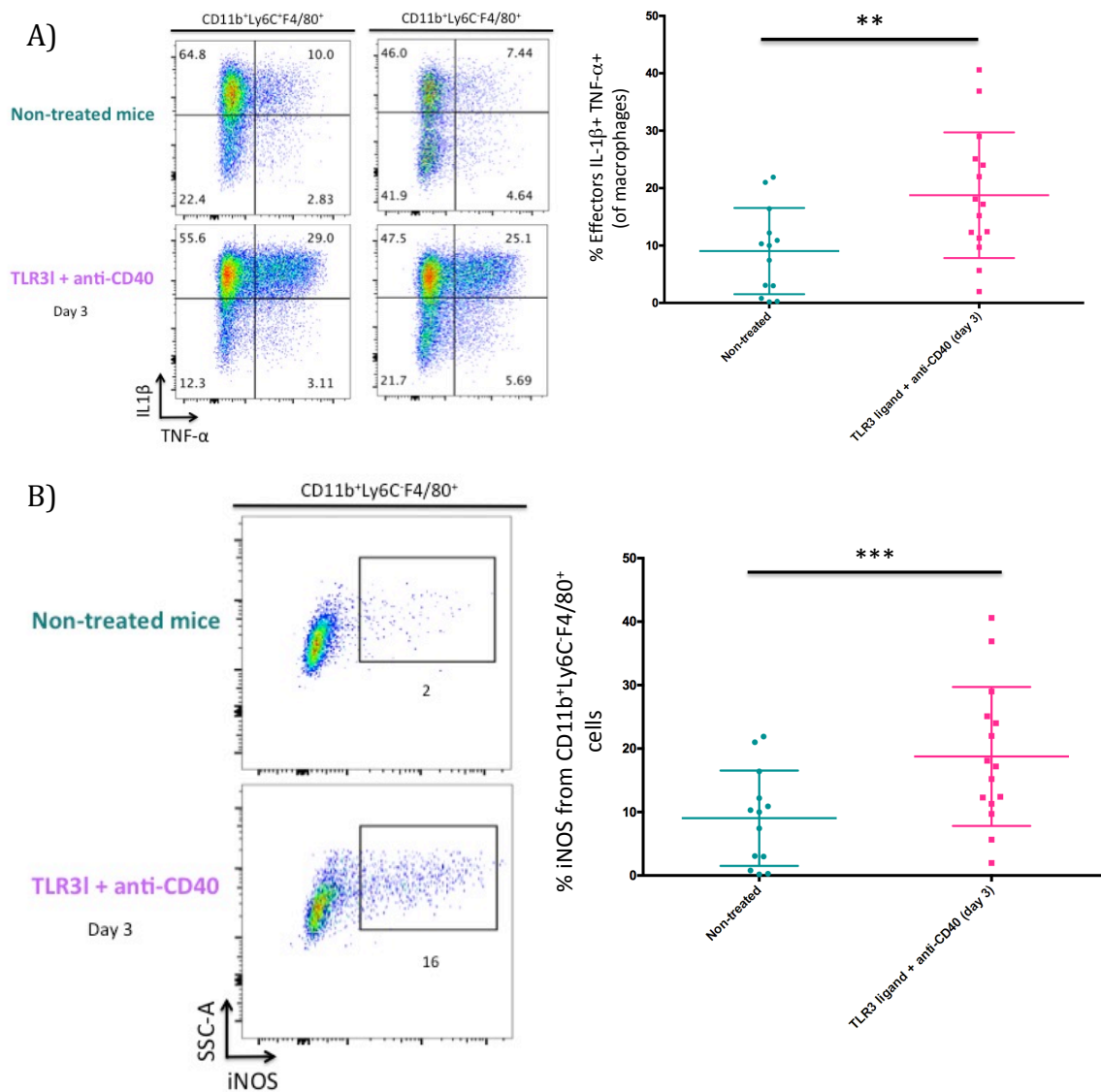increase in the production of iNOS on treated compared to non-treated mice (Figure 11B).



**Figure 11: Upregulation of the expression of IL-1$\beta$, TNF-$\alpha$ and iNOS by macrophages. (A)** Representative FACS plots (left) and respective graphic (right) from tumours of non-treated and treated mice, seventy-two hours after treatment showing the frequency of IL1$\beta$, TNF-$\alpha$ (CD11b$^+$ Ly6C$^{(+/-)}$ F4/80$^+$) and **(B)** iNOS by macrophages (CD11b$^+$ Ly6C$^-$ F4/80$^+$). Statistical analysis was performed using the non-parametric Mann-Whitney test.

Paralleled to this, the expression of the immunosuppressive marker PD-L1 was also assessed. We found that there was a reduction in the percentage of myeloid cells (CD11b+ cells) expressing high levels of PD-L1 within regressing compared to progressing tumours (Figure 12A; Supplementary figure 4). These results, which are consistent to what Miguel Pinto showed in his thesis (Pinto, 2017), indicate that the treatment leads to a change in the myeloid cell functions towards a more anti-tumoural phenotype.

These results led us to assess the role of these pro-inflammatory cytokines and enzymes in the response to the treatment with TLR3/CD40.

40

A)
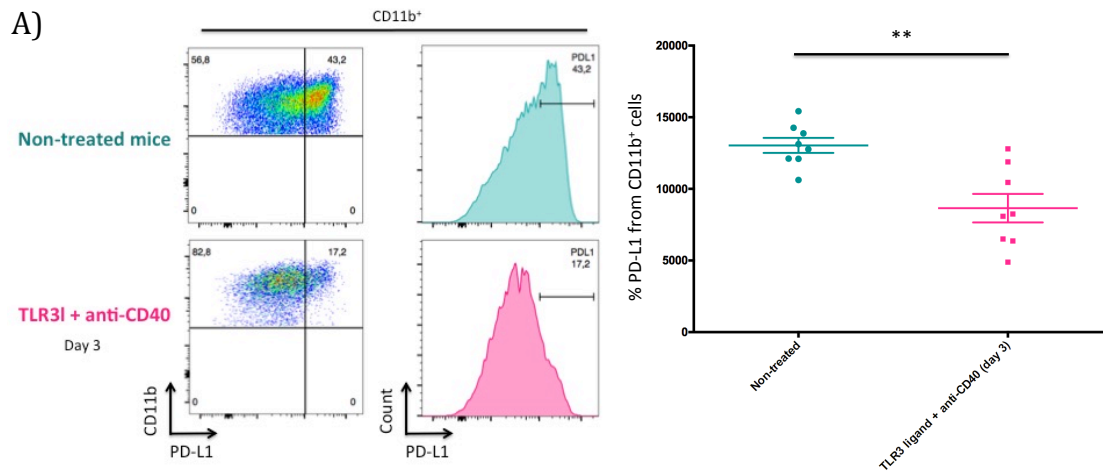


**Figure 12: Downregulation of the expression of PD-L1 upon treatment. (A)** FACS plots (left) and respective graphic of the percentage of PD-L1 (right) from tumours of non-treated and treated mice seventy-two hours after the treatment. Statistical analysis was performed using the non-parametric Mann-Whitney test.

# 6. Role of IL-1β, TNF-α and iNOS in response to TLR3/CD40

To understand the direct effect of TLR3/CD40 on macrophages we used BMDM. Hence, we measured gene expression on BMDM that were polarized in various conditions during 24 hours. We compared M1, M2a, M2c conditions and also in the presence of TLR3/CD40. While *Il-1β* was upregulated by TLR3/CD40, *Nos2* messenger appears selective to M1 condition. *Tnf-α* was upregulated both in M1 condition and by TLR3/CD40 (Figures 13A-C).
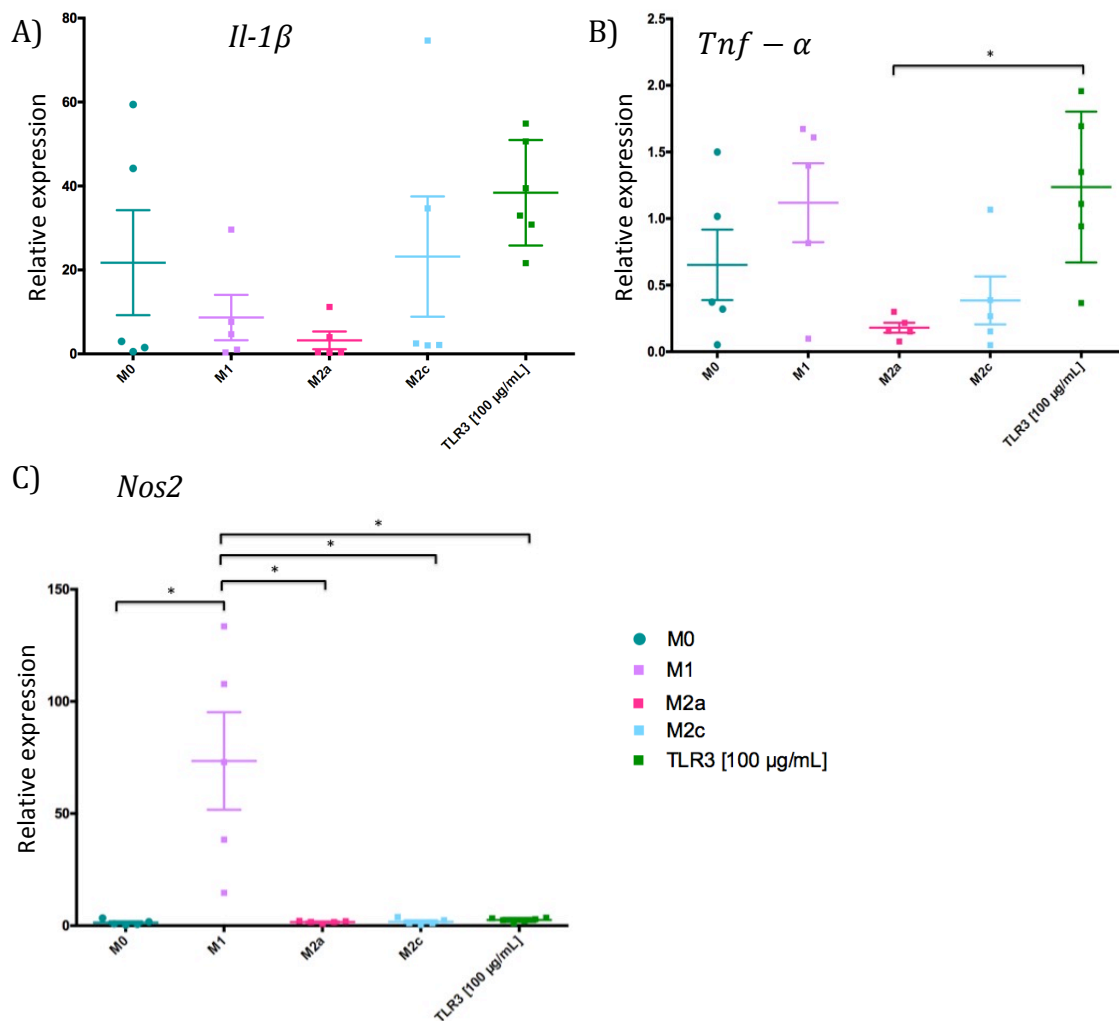
**Figure 13: Gene expression in BMDM. (A)**, **(B)** and **(C)** Respective gene expression of *Il-1β*, *Tnf-α* and *Nos2* expression in polarized and treated macrophages that were in contact with E0771 tumour cells during 24 hours (M0, M1, M2a and M2c, n=5; TLR3/CD40 n=6). *β2 microglobulin* was used as endogenous reference and statistical analysis was performed using Tukey's multiple compared test.

We also went on to measure iNOS protein levels by intracellular FACS staining on BMDM polarized in various conditions. This analysis showed that the proportion of iNOS-expressing BMDM were induced at much higher levels in M1 compared to TLR3/CD40-stimulated macrophages. Nevertheless, although to a much lesser extent, iNOS was also induced in TLR3/CD40-stimulated macrophages (Figure 14A).
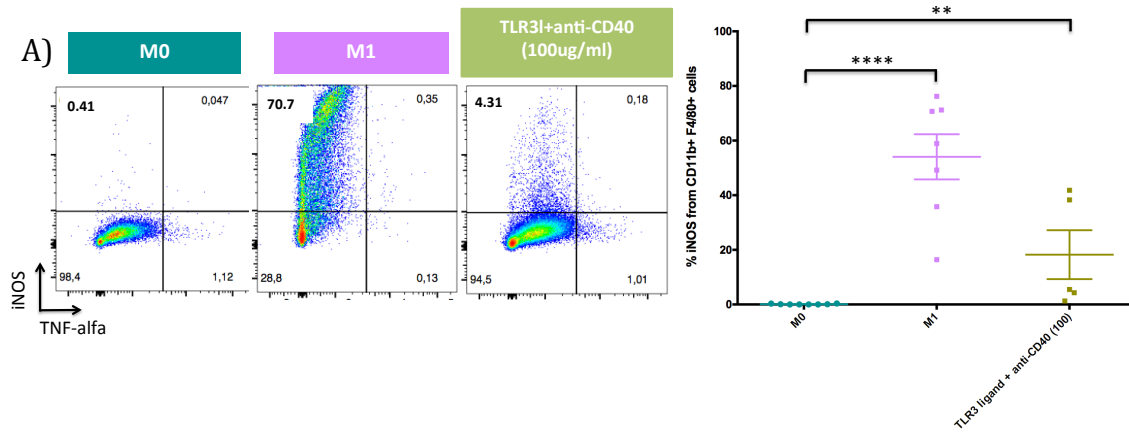
**Figure 14: Upregulation of iNOS in M1 macrophages. (A)** Representative FACS plots (right) and graphic (right) from polarized and treated macrophages. Statistical analysis was performed using Tukey's multiple compared test.

In order to decipher which factor mediates the anti-tumour effect of the treatment, we decided to block IL-1$\beta$, TNF-$\alpha$ and iNOS production by using agents already reported to work *in vivo*. We found that, while IL-1β and iNOS are dispensable for tumour eradication, TNF-$\alpha$ is required for the induction of tumour regression by TLR3/CD40 because this treatment does not work when the production of TNF-$\alpha$ is neutralized (Figures 15A-D).

Further investigation is required to determine the specific role of TNF-$\alpha$. This effector molecule could impact directly the tumour cells by preventing proliferation or even induce cell death. Macrophages activated *in vivo* with TLR3/CD40 could also act on the tumour microenvironment through, for instance, induction of T cell responses against tumour and/or inhibition of angiogenesis.
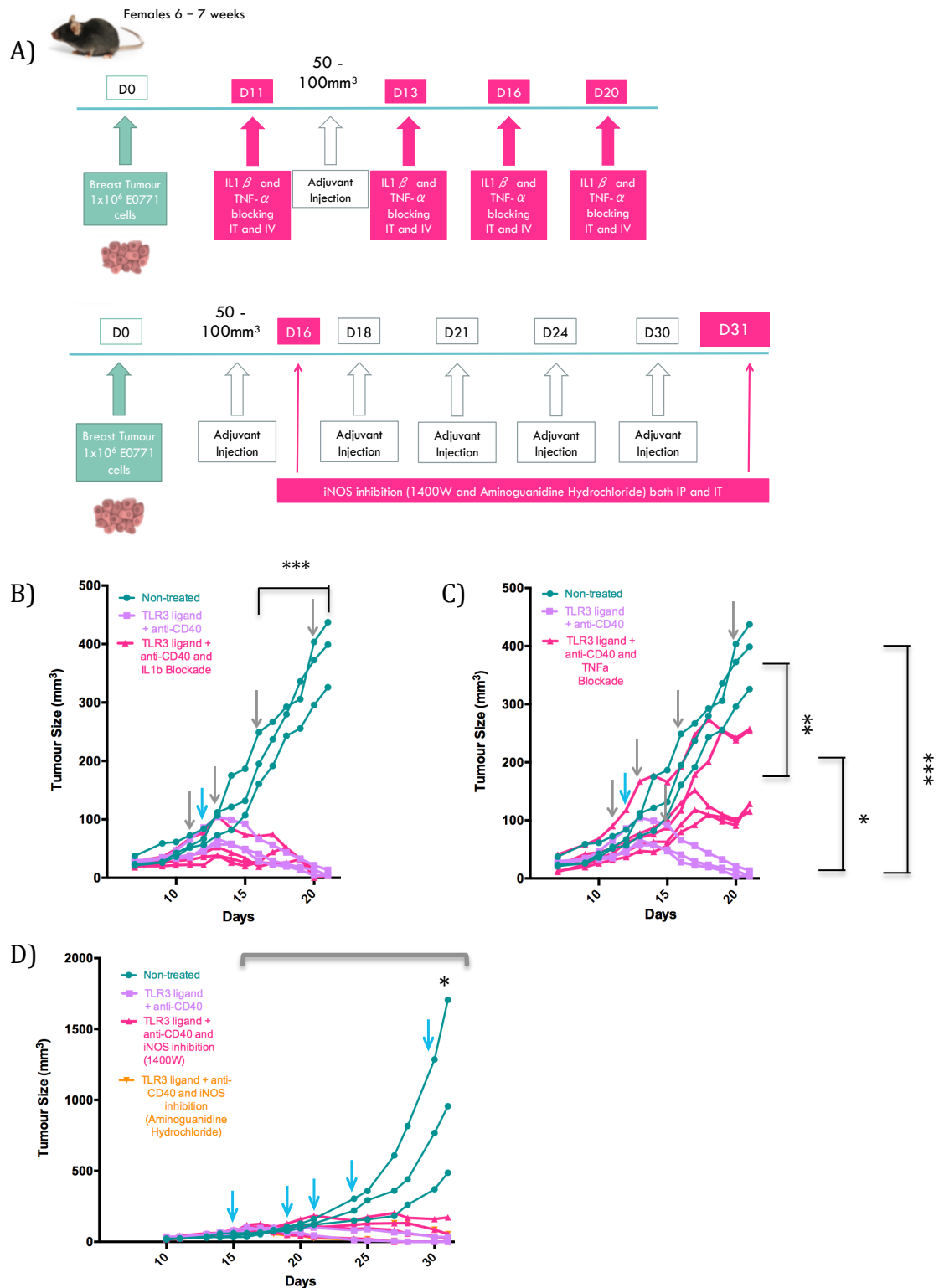
**Figure 15: Role of IL-1β, TNF-α and iNOS upon treatment**. **(A)** Schematic representation of the schedule for tumour injection (green), adjuvant injection (grey line) and blocking of IL-1β, TNF-α and inhibition of iNOS (pink). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=3), treated mice with TLR3/CD40 (purple squares; n=3) and treated mice with TLR3/CD40 and IL-1β neutralization (pink triangles; n=4). On days 11, 13, 16 and 20 (grey arrows) mice were injected with the IL1β blocker, both IT and IV and on day 12 (blue arrow), mice were injected with TLR3/CD40 treatment. **(C)** E0771 tumour growth in non-treated mice (blue circle; n=3), treated mice with TLR3/CD40 (purple squares; n=3) and treated mice with TLR3/CD40

and TNF-$\alpha$ neutralization (pink triangles; n=5). On days 11, 13, 16 and 20 (grey arrows) mice were injected with the TNF-$\alpha$ blocker, both IT and IV and on day 12 (blue arrow), mice were injected with TLR3/CD40 treatment. **(D)** E0771 tumour growth in non-treated mice (blue circle; n=3), treated mice with TLR3/CD40 (purple squares; n=2), treated mice with TLR3/CD40 and 1400W iNOS inhibitor (pink triangles; n=4) and treated mice with TLR3/CD40 and Aminoguanidine Hydrochloride iNOS inhibitor (yellow triangles; n=2). Since day 16 (grey line), mice were injected every day with the iNOS inhibitor, both IP and IT and on days 15, 18, 21, 24 and 30 (blue arrows), mice were injected with TLR3/CD40 treatment. Statistical analysis was performed using Tukey's multiple compared test.

# 7. *In vivo* re-challenge of TLR3/CD40 treatment and immunologic memory

We also went to determine if the tumour regression induced by TLR3/CD40 was long lasting and could prevent "experimental" relapses. Thus, after tumour complete eradication, we waited about 20 days and mice received a second injection of $1 \times 10^6$ E0771 tumour cells on the contralateral side of the first injection (Figure 16A). We found that tumour cells were not able to establish a tumour mass, showing that tumour-free survivors were resistant to tumour re-implantation and indicating the generation of a long-lasting adaptive immunity against E0771 tumour cells (Figure 16B). This is consistent with the results already shown by Miguel Pinto in his thesis (Pinto, 2017).
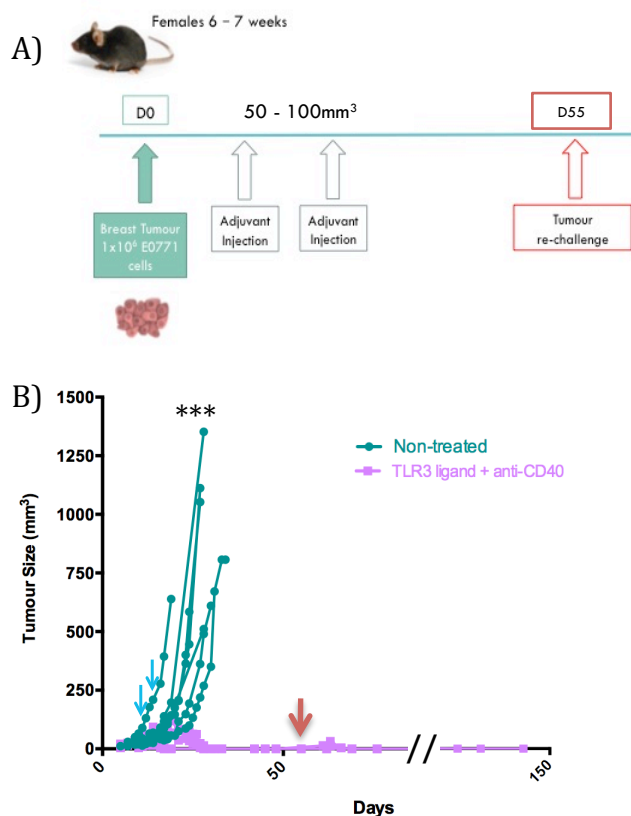
**Figure 16: Long-lasting adaptive immunity by tumour-free survivors. (A)** Schematic representation of the schedule for tumour injection (green), adjuvant injection (grey line) and re-challenge (red line). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=7), treated mice with TLR3/CD40 (purple squares; n=7) on days 18 and 21 (blue arrows). Mice were re-injected on day 55 (red arrow). Statistical analysis was performed using the non-parametric

Mann-Whitney test.

# 8. Required immune cells for tumour complete eradication upon treatment

Given that the treatment with TLR3/CD40 led to long-lasting memory and protected against novel tumour challenge, CD8+ T cells could be the key players in the complete eradication of the tumour. To assess the role of CD8+ T cells we used a depleting anti-CD8 mAb. Thus, tumour-bearing mice were depleted in CD8+ T cells and then received TLR3/CD40 treatment (Figure 17A).

CD8+ T cell depletion was confirmed by blood analysis using FACS on the day of the first treatment (Supplementary figure 5).

Consistent with the results already shown by Miguel Pinto in his master thesis (Pinto, 2017), the treatment failed to induce tumour regression when CD8+ T cells are depleted, indicating that CD8+ T cells are responsible for tumour eradication upon treatment (Figure 17B).

Importantly, between days 13 and 18, the treatment induced a control in tumour growth in a CD8+ T cell-independent manner. This suggests that early after treatment macrophages are likely the effectors controlling tumour progression while after one week the CD8+ T cells are the effectors required to eliminate the tumour. Altogether, these results suggest a two-step effect of the TLR3/CD40 treatment. First, macrophages get activated and can limit tumour expansion through direct or indirect tumour killing. Second, they may also participate in the priming of CD8+ T cells, which in turn will induce complete tumour elimination.
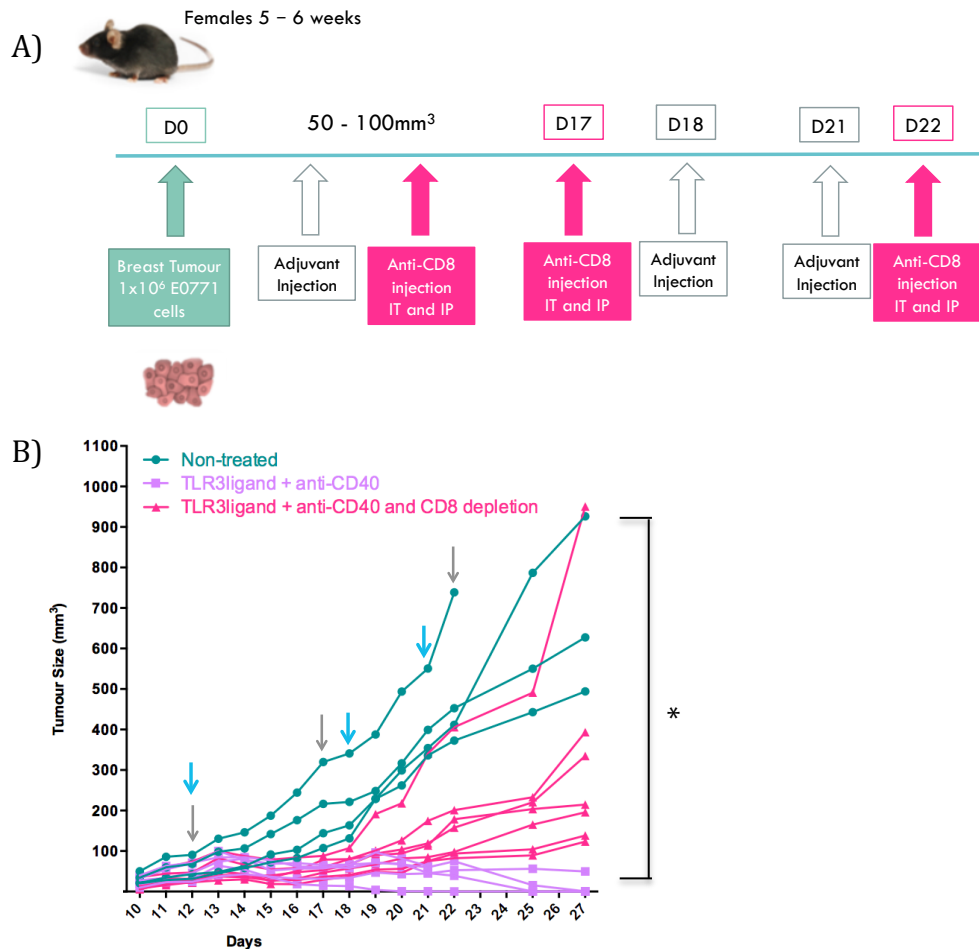
**Figure 17: CD8+ T cells are necessary for tumour eradication upon TLR3/CD40 intra-tumour injection. (A)** Schematic representation of the schedule for tumour injection (green), adjuvant injection (grey line) and depleting anti-CD8 (pink) injection both IT and IP every five days. **(B)** E0771 tumour growth in non-treated mice (blue circle; n=4), treated mice with TLR3/CD40 (purple squares; n=4) on days 12, 18 and 21 (blue arrows) and treated mice with TLR3/CD40 and depleted on CD8+ T cells with anti-CD8 mAb (pink squares; n=7) on days 12, 17 and 22 (grey arrows). Statistical analysis was performed using Tukey's multiple compared test.

# 9. Effector functions of CD8+ T cells upon treatment with TLR3/CD40

To determine the effector functions of CD8+ T cells, the production of TNF-$\alpha$ and IFN-$\gamma$ was assessed by FACS. Consistent with the results already shown by Miguel Pinto in his master thesis (Pinto, 2017), we found that treated mice were capable of producing high amounts of these effectors in comparison to non-treated mice. These results demonstrate that CD8+ T cells responded to the treatment and upregulated anti-tumour effector functions (Figure 18).
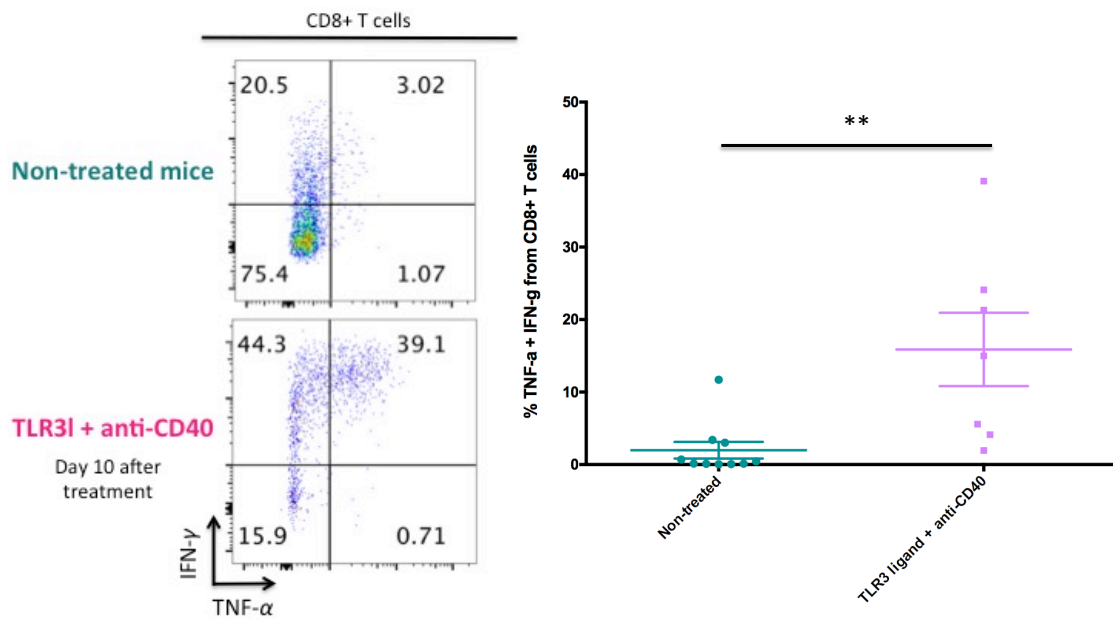
**Figure 18**: **TLR3/CD40 treatment increases anti-tumour CD8+ T effectors in the tumour.** Representative FACS plots (left) and graphic (left) from tumours of non-treated and treated mice, showing the frequency of TNF-$\alpha$ and IFN-$\gamma$ by CD8+ T cells ten days after treatment with TLR3/CD40. Statistical analysis was performed using the non-parametric Mann-Whitney test.

IFN-γ was also found to be critical for the treatment-induced tumour regression as TLR3/CD40 ligand plus anti-CD40 failed to induce tumour growth delay or tumour regression in IFN-$\gamma$ $^{-/-}$ mice (Figure 19A-B).
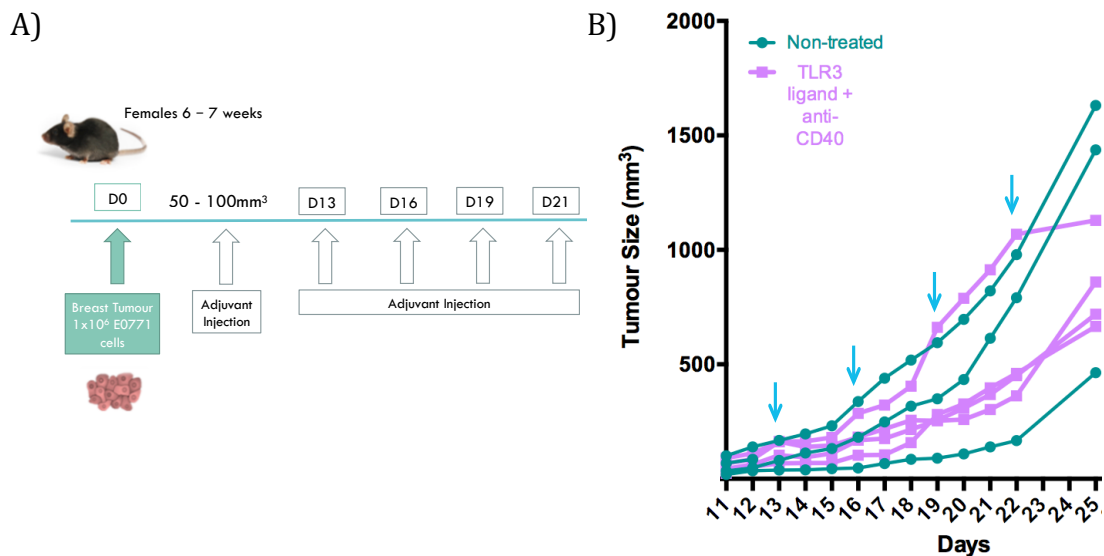
A)



B)



**Figure 19**: **IFN-$\gamma$ is important for the treatment to work. (A)** Schematic representation of the schedule for tumour injection (green) and adjuvant injection every three days, since day 13 (grey line). **(B)** E0771 tumour growth in non-treated (blue circle; n=4) and treated mice with TLR3/CD40 (purple squares; n=4) in IFN-$\gamma^{-/-}$ mice. Mice were injected with TLR3/CD40 IT every three days, since day 13 (blue arrows). Statistical analysis was performed using the non-parametric Mann-Whitney test.

# 10. Indirect pathway between macrophages and CD8+ T cells

Until now, we have evidence that in a mouse model of mammary tumour, macrophages respond to the combination of TLR3/CD40 and that tumour eradication is mediated by CD8+ T cells. This led us to question the role of cells that are critical to cross-present tumour antigens to CD8+ T cells. In particular, IT CD103+ DCs have been identified as active APCs in the tumor microenvironment that process antigens and are crucial to the induction of an anti-tumoural T-cell response. These CD103+ DCs have a unique property to cross-present antigens to CD8+ T cells. The development of these DCs has been shown to rely on the transcription factor BATF3. Although we never observed differences between the accumulation of CD103+ DCs in the tumour upon treatment (data not shown) we decided to determine their role upon treatment. To do so, we treated tumour-bearing role of BATF3 $^{-/-}$ mice, which lack the so-called CD103+ BATF3+ DC1.

We found that upon TLR3/CD40 IT injection, the treatment failed to induce tumour regression, suggesting that this type of DCs might have a role in CD8+ T cell activation (Figures 20A-B).
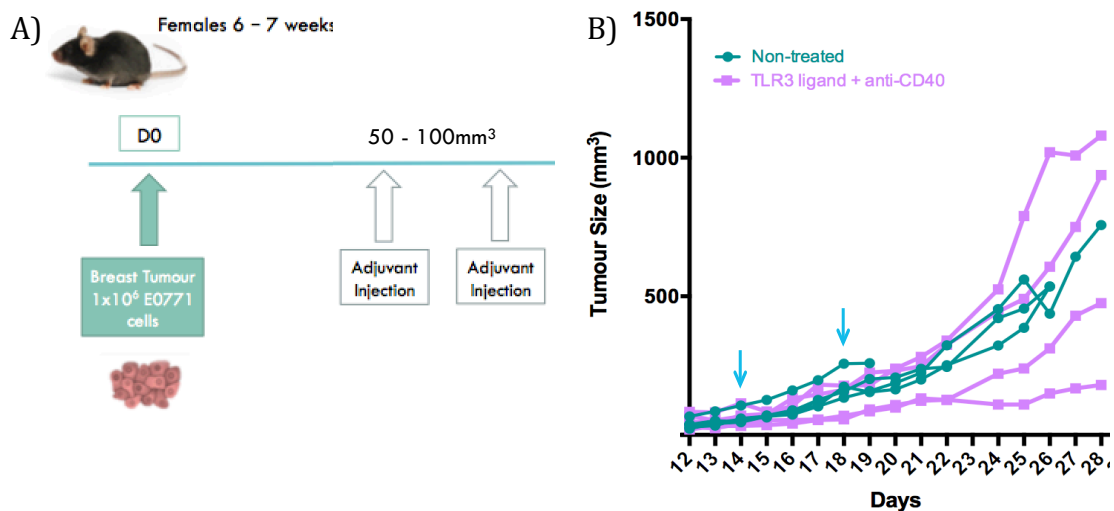


**Figure 20: Dendritic cells are important in tumour eradication upon treatment with TLR3/CD40. (A)** Schematic representation of the schedule for tumour injection (green) and adjuvant injection (grey line). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=4) and treated mice with TLR3/CD40 IT (purple squares; n=4) on days 14 and 18 (blue arrows). Statistical analysis was performed using the non-parametric Mann-Whitney test.

# 11. *In vivo* anti-tumour effect of other TLR ligand in combination with anti-CD40 and dependency on neutrophils

In analogy with macrophages, TANs can exert pro-tumoural as well as anti-tumoural functions (Fridlender et al., 2009; Mantovani, 2009; Mantovani et

al., 2011). Thus, to understand if a specific TLR ligand plus anti-CD40 was generating an anti-tumour response dependent on neutrophils, this myeloid subset was depleted with anti-Gr1 every three to five days (Figure 21A).

We found that TLR4 ligand plus anti-CD40, henceforth called as TLR4/CD40, failed to induce tumour regression upon neutrophil depletion (Figure 21B). Importantly, neutrophils are dispensable for the elimination of tumour triggered by the other TLR ligands (Supplementary figure 6A-B), suggesting that TLR4/CD40 is the only one that is capable of inducing tumour regression in a neutrophil-dependent manner.

These results suggest that the local signaling pathways capable of shaping macrophage and neutrophil responses to the tumour are different and non-overlapping.
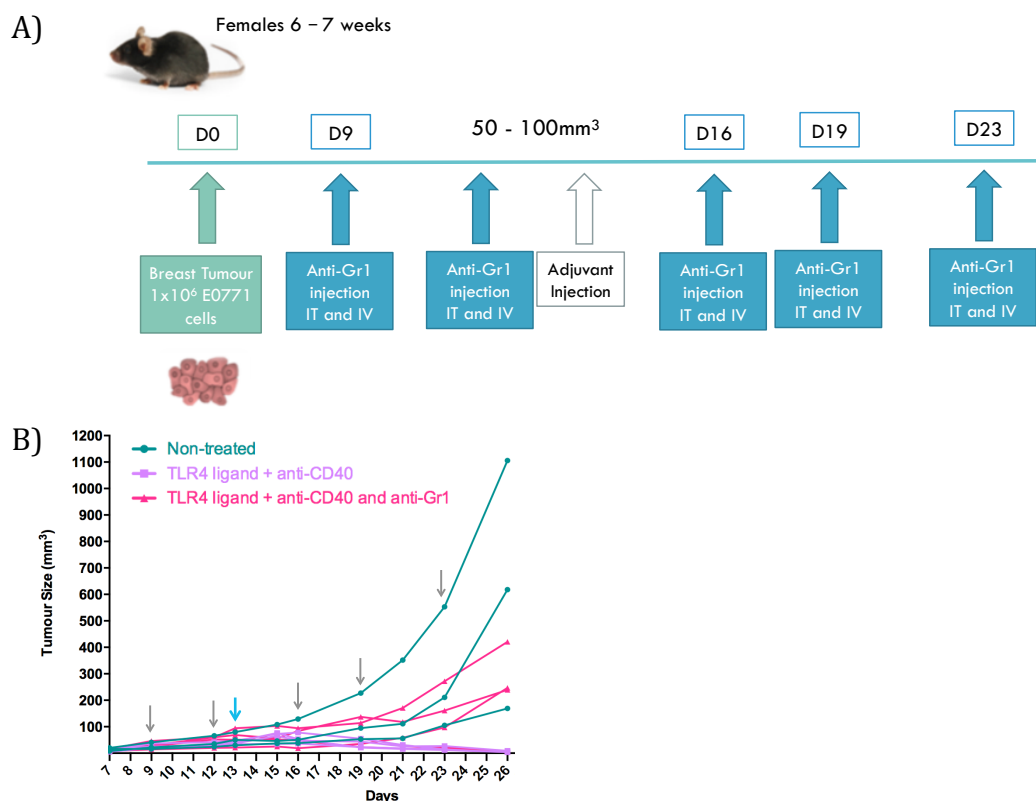


**Figure 21:** *In vivo* **TLR4/CD40 anti-tumour effect depends on neutrophils. (A)** Schematic representation of the schedule for tumour injection (green), anti-Gr1 injection both intra-tumour and intravenously every 3 to 5 days (blue) and adjuvant injection (grey line). **(B)** E0771 tumour growth in non-treated mice (blue circle; n=3), treated mice with TLR4/CD40 (purple squares; n=3) and treated mice with TLR4/CD40 and depleted on neutrophils with anti-Gr1 (pink triangles; n=3). On days 9, 12, 16, 19 and 24 (grey arrows) mice were depleted on neutrophils both IV and IP. On day 13 (blue arrow), mice were injected with TLR4/CD40 IT. Statistical analysis was performed using Tukey's multiple compared test.

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# CHAPTER 5: DISCUSSION

Nowadays, there is still a lack of information characterizing the anti-tumour functions, phenotype and transcriptional programs of myeloid cells. Consequently, our work aimed to study these cells in the context of tumour responses. For that, we successfully found *in vivo* conditions to induce macrophages and neutrophils to perform anti-tumour functions upon stimulation with TLR3 or TLR4 ligand both in combination with agonist anti-CD40 mAb, respectively. This anti-tumour phenotype consistently led to tumour complete remission in the majority of treated mice.

We explored the effects of TLR3/CD40 stimulation and found that, although macrophages produced pro-inflammatory cytokines, such as TNF-$\alpha$ and IL-1$\beta$, and high amounts of iNOS, only TNF-$\alpha$ is required for tumour remission. Moreover, tumour-free survivors were resistant to tumour re-implantation indicating a long-lasting adaptive immunity that was dependent on CD8+ T cells. Regarding the treatment with TLR4/CD40, further studies need to be done in order to dissect the mechanisms behind the potentiation of anti-tumour neutrophils.

In this chapter we will discuss the experimental limitations of our model, as well as propose alternative approaches. Afterwards we will hypothesize on the mechanisms involved in our model, discuss future work and conclude with the implications of our findings.

## 1. Experimental limitations

In this thesis we used an orthotopic mouse transplanted tumour model. These transplantable mouse models have permitted great discoveries in the oncoimmunology field. An example is the identification of immune checkpoints and the development of the immune checkpoint blockers. The importance of these discoveries is demonstrated by the number of patients, whose prognosis was very unfavorable, and who have had their lives prolonged of many years. The attribution of the Nobel Prize of Physiology and Medicine 2018 to James Allison and Tasuku Honjo clearly exemplified the remarkable outcome of discoveries that were first observed in transplantable tumour mouse models.

However, this transplantable tumour approach system has a main weakness as it bypasses the tumorigenesis phase and the long-term establishment and modulation of the immune response as it occurs in humans. Transplanted tumour progression lacks many features of natural carcinogenesis and they also grow very rapidly, which are critical differences with human tumours (Ngiow, Loi, Thomas, & Smyth, 2016; Sanmamed, Chester, Melero, & Kohrt, 2016). It is well known that most of immunotherapies fail to provide

similar effects in humans compared with mice. In fact, the rate of successful translation from animal models to clinical trials has been estimated to be less than 8% (Mak, Evaniew, & Ghert, 2014). For all these reasons, we plan to modulate myeloid cells in chemically-induced and oncogene-driven tumour mouse models. These models are much more reliable in terms of tumour development and the constitution of the myeloid compartment should be more representative of naturally developed tumours.

## 2. TLR3/CD40 treatment acts on macrophages and leads to tumour complete remission

Currently, the potential of TLR agonists to induce effective immune responses against tumours has been widely studied, and they are proposed to be used as adjuvant in cancer immunotherapy (Kaczanowska, Joseph, & Davila, 2013; K. Li, Qu, Chen, Wu, & Shi, 2017; Shi et al., 2016). We attempted to dissect the effect of TLR3 ligand on myeloid cells and its effect on the tumour immune response.

One of our objectives was to determine if the anti-tumour effect of TLR3/CD40 was dependent on the cytokines and enzymes produced by macrophages - IL-1$\beta$, TNF-$\alpha$ and iNOS.

The role of IL-1 $\beta$ in cancer is controversial. It is a pleiotropic cytokine that in one hand can act as pro-tumoural and induce tumor growth, angiogenesis and consequently metastasis (Elaraj et al., 2006; Lewis, Varghese, Xu, & Alexander, 2006; Voronov et al., 2002) or in another hand, can act as anti-tumoural by increasing antigen-presentation or promoting CD8+ T cell activation (Ghiringhelli et al., 2009; Yao et al., 2017).

As mentioned in section 5 of the introduction, macrophages can also be cytotoxic and kill tumour cells through the production of soluble factors, such as NO or TNF-$\alpha$.

TNF-$\alpha$ is well-described as a potent pro-inflammatory cytokine known to participate in tumour cell killing (Blankenstein et al., 1991; Urban, Shepard, Rothstein, Sugarman, & Schreiber, 1986). For instance, low-doses of TNF- $\alpha$ can improve the stabilization of blood vessels and tumour perfusion, while it enhances tumour specific immune responses by an increase in T cell infiltration and OS, that is exclusively mediated by CD8+ T cells (Johansson, Hamzah, Payne, & Ganss, 2012). The binding of the TNF- $\alpha$ trimer to TNFR1 causes trimerization of TNFR1, resulting in the formation of a complex formed by TNFR1, TRADD, TRAF2 and RIP that when sustained leads to JNK activation and contributes to tumour cell death (pro-apoptotic pathway) (Wang & Li, 2008). In addition, Kratochvill *et al*. also showed that TNF- $\alpha$ is essential for blocking M2 macrophages in the TME (Kratochvill et al., 2015).

In tumours, NO has been described to have both pro- and anti-tumoural effects. This dichotomy can be explained by the complexity of signaling

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pathways in tumour cells that can respond to NO in very different ways mainly depending on its concentrations (Rahat & Hemmerlein, 2013). Hypoxia conditions and immunossupressive cytokines can inhibit iNOS activity and lead to low production of NO, supporting tumour growth and metastasis formation. In contrast, NO can also act as an important mediator involved in tumour cell killing (Klimp et al., 2002; MacMicking, Xie, & Nathan, 1997). High levels of NO may lead to tumour apoptosis by enhancing cytochrome c release from the mitocondria and activating caspases. In addition, it was already shown that danger signals could induce iNOS+ macrophages that are critical to tumour rejection. For instance, after low-dose gamma radiation, M2 macrophages can be polarized into M1, leading to the production of NO by macrophages. This NO-producing macrophages where shown to be critical for tumour rejection by the production of Th1 chemokines leading to the recruitment of effector T cells and suppression of angiogenic factors (Klug et al., 2013).

After neutralizing the production of each factor, IL-1$\beta$, TNF-$\alpha$ and iNOS, *in vivo* we found that TNF-$\alpha$ played a key role in tumour eradication.

*In vitro* killing assays done by Henning Boekhoff in the laboratory using M1-polarised or TLR3/CD40 stimulated macrophages suggested that the M1 conditioning medium (supernatant of polarized M1 macrophages) induced higher tumour cell killing compared of TLR3/CD40 conditioning medium. By contrast, when co-cultured with tumour cells, TLR3/CD40 stimulated macrophages had a higher potential to induce tumour cell killing than M1-polarised macrophages. This led us to hypothesize that M1 macrophages might produce a soluble factor (possibly NO) while TLR3/CD40 stimulated macrophages might act through direct cell contact to kill tumour cells (data not shown).

To assess this we used iNOS inhibitor drugs that are widely recognized to inhibit iNOS. However, we found only limited diminution of NO production from either M1 macrophages or TLR3/CD40 stimulated macrophages. Thus, to definitively determine the potential role of iNOS, we prepared BMDM from iNOS -/- mice. To our surprise, while NO species were not detected (with a Griess assay) in the supernatant of M1 or TLR3/CD40 activated macrophages, tumour killing was only partially inhibited consistent with a mild role for NO (and thus iNOS) as a mechanism leading to tumour cell control *in vivo* (data not shown).

To further dissect the mechanism of action of tumour killing by these BMDM we also plan to use transwells where tumour cells will be plated in the bottom and in the upper chamber the macrophages will be added. A permeable membrane will be separating both tumour cells and macrophages and after an incubation period, we will stain and count the cells in order to see if macrophages had killed the tumour cells. From this experiment we expect to have tumour cell death due to the secretion of other soluble factors (e.g.

granzyme, ROS, or even a combination of everything) by macrophages that work to limit tumour growth.

Contact-dependent tumour cell-killing might depend on the expression of TNF-related apoptosis-inducing ligand (TRAIL), NKG2D or the first apoptosis signal ligand (FasL) by macrophages (Baba et al., 2008; Herbeuval et al., 2003; Klimp et al., 2002; Z. Zhou et al., 2012). We have assessed the expression of cytotoxic molecules by macrophages by real-time PCR. However, none of the markers we have assessed were selectively expressed by TLR3/CD40-stimulated macrophages, leaving the contact-dependent tumour cell-killing mechanism still unresolved (data not shown).

Macrophages are also able to phagocyte tumour cells and one of the mechanisms involves the recognition of tumour cells that express CD47 via expression of SIRP$\alpha$ on macrophages, the "don't eat me" signal. In order to test this *in vivo*, we plan to use a SIRP$\alpha$-blocking antibody, and see if the TLR3/CD40 induces tumour regression. *In vitro*, we plan to do a phagocytosis assay where fluorescent E0771 tumour cells are incubated with activated BMDM and then we assess the level of fluorescence in macrophages by fluorescence microscopy.

Macrophages can also be anti-angiogenic depending on the stage of the tumour growth and the microenvironmental conditions (Herbeuval et al., 2003; Klimp et al., 2002; C. F. Nathan, 1987). For instance, in the presence of hypoxia, tumours can also secrete GM-CSF, which have been shown to stimulate macrophages to produce IL-12 and a soluble variant of the VEGF receptor 1 (sVEGFR1) in an hypoxia-inducible factor (HIF) 2α-dependent manner, which will block VEGF activity and therefore suppress angiogenesis (J. Roda et al., 2012). These anti-angiogenic macrophages have already been described to inhibit breast cancer growth and metastasis and also decrease tumor growth in melanoma mice models (Eubank et al., 2009; J. Roda et al., 2012; J. M. Roda et al., 2012). Other studies blocking IL-4 or even the injection of TLR9 ligand in combination with an antibody specific for the IL-10 receptor into tumour-bearing mice can shift TAMs from an M2 to a M1 phenotype (Guiducci, Vicari, Sangaletti, Trinchieri, & Colombo, 2005; Linde et al., 2012). All findings about these anti-angiogenic properties of macrophages show that, when properly instructed, they can target and dissociate the tumour-associated vasculature.

In order to test this hypothesis, we intend to take advantage of *in vitro* and *in vivo* approaches. We will use M1-polarized and TLR3/CD40 treated BMDM and do gene expression to test some angiogenic markers, such as VEGF, angiopoietin or the platelet-derived growth factor (PDGF). Immunohistochemistry, transmigration and scratch assays are also other options that can be used to understand the activity of these macrophages upon treatment *in vitro*. Secretion of pro- or anti-angiogenic factors by BMDM stimulated in various conditions will also be assessed using a chick embryo model, in particular its chorioallantoid membrane (CAM). By using some anti-

angiogenic inhibitors, such as bevacizumab, sunitinib or rapamycin that are already in the clinic, we could also see if TLR3/CD40 treatment induces tumour regression.

It is plausible that anti-tumour macrophages (through either their anti-angiogenic, cytotoxic or other unidentified functions) can also participate in DC stimulation that in turn can activate CD8+ T cells. Although Gilfillan and her team already showed that DC1s are not essential for CD8 responses induced by TLR3 ligand immunotherapy in B16 melanoma tumours (Gilfillan et al., 2018), there are other works that emphasize the importance of DCs in priming T cells and promoting anti-tumour responses (Ruffell et al., 2014). In fact, in this thesis we show that BATF3, which is important for the development and function of DC1s, is also important in the response to TLR3/CD40.

In sum, TLR3+CD40 treatment generates anti-tumour macrophages, however, the identification of their anti-tumour functions still needs to be elucidated.

All these results lead us to build our two-step model where the treatment activates macrophages that, in the beginning can act as potent phagocytic killers, secrete soluble factors or even prevent blood supply, leading to a delay in tumour growth. Then, these activated macrophages will, in a direct or indirect way (with the help of DCs), activate CD8+ T cells, which will cause a complete remission of the tumour (Figure 22).
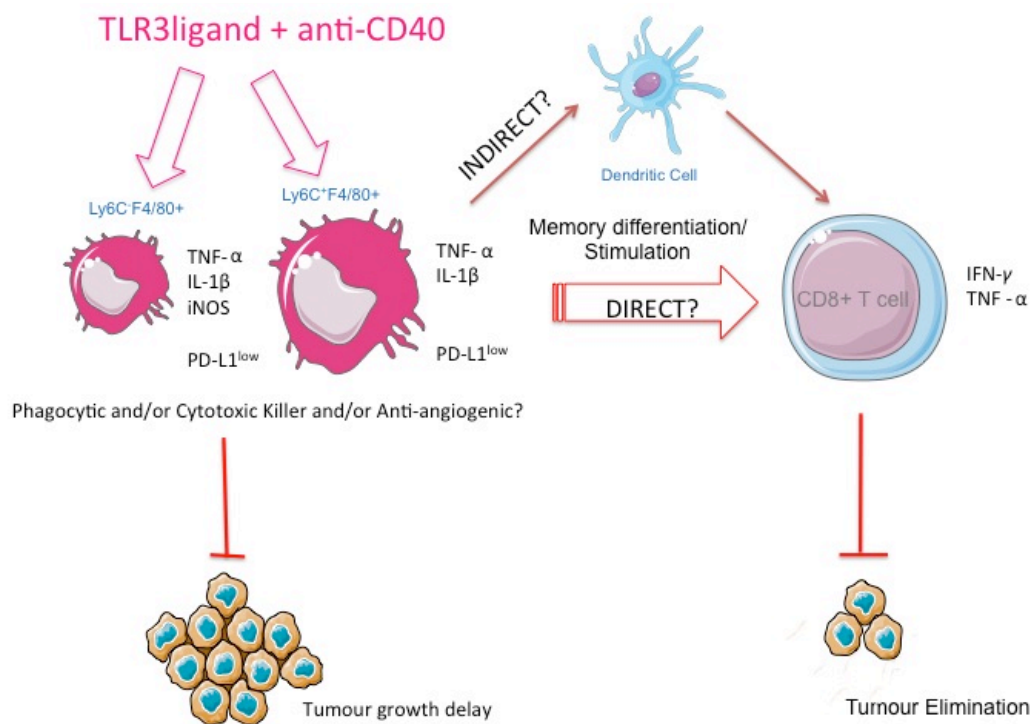


**Figure 22: Representation of our proposed two-step model.**

# 3. *In vivo* TLR4/CD40 anti-tumour effect depends on neutrophils

An important goal of this thesis was to assess whether other maturing agents also had the potential to change other myeloid cell effectors, in particular neutrophils. Preliminary data showed that upon neutrophil depletion using anti-Gr1 depleting Abs, the treatment with TLR4/CD40 was abolished. This suggested that, in response to TLR4/CD40 treatment, the critical myeloid cell compartment is neutrophil.

In the future, we intend to further characterize neutrophil anti-tumour functions upon TLR4/CD40 treatment. Additionally, we also want to test this treatment in different mouse tumour models (transplantable colon cancer and triple negative breast cancer cell lines - CT26 and 4T1, respectively - in BALB/c, and a melanoma model - B16F10 - in C57BL/6J), similarly to what my colleague Miguel Pinto did for his thesis using TLR3/CD40 (data not shown) (Pinto, 2017). With this we could understand if the stimulation through TLR4 induces tumour regression in various tumour types.

## 4. Long-term goal of our findings

To ensure that macrophages are the key myeloid players involved in TLR3/CD40 treatment we will use LysM-cre CFS1R-Floxed DTR CX3CR1-GFP mice (recently received from Dr. Ana Domingos (IGC, Oeiras, Portugal)) that allow selective macrophage depletion upon injection with diphteria toxin. In the same way to confirm the exclusive role of neutrophils, these mice will be used to assess tumour growth in response to TLR4/CD40 treatment.

In order to decipher some of the questions of our proposed two-step model, we also plan to do immunohistochemistry on tumour samples that have received the TLR3/CD40 treatment. This will help us to understand the localization of specific cells with the TME. For instance, based on our two-step model, we believe that macrophages might be closer to T cells on treated samples. Something that we also want to assess is to whether there are more blood vessels in non-treated mice compared to treated ones. This can also be extended to neutrophils upon treatment with TLR4/CD40. As shown in supplementary figure 7, the technique is already optimized for all the markers and now we intend to increase our number of samples to have statistically relevant results.

In addition, an important unanswered question is as to whether macrophages are locally reprogrammed or if monocyte-derived macrophages are recruited and polarized *in situ* by the treatment. To address this question, we are establishing collaboration with Dr. David Withers (Birmingham, UK) to take advantage of a photo convertible fluorescent protein "Kaede"- transgenic mice. Kaede changes from green to red upon exposure to violet light (Tomura et al., 2008). Thus, in these mice all cells appear green, the tumour can be exposed to

violet light inducing all host and tumour-infiltrating immune cells to become red just before the application of the treatment. This way it is possible to distinguish between the response of Kaede-red resident and Kaede-green migrating immune cells. From this model, we expect to see green cells within the tumour, meaning that we have a recruitment of monocytes that will give rise to macrophages upon treatment with TLR3/CD40.

A long-term goal is to decipher the transcriptional program(s) that supports *in vivo* anti-tumour myeloid cell differentiation. Both anti-tumour macrophages and neutrophils will be analyzed with genome-wide unbiased approaches - transcriptomics. To dissect the diversity of effectors within the macrophage and neutrophil compartments we will perform RNA-sequencing at the single cell level. This will allow a clear understanding as to whether cells share anti-tumoural functions or if there is a clear partition in labour. If the anti-tumour responses are segregated in exclusive macrophage and neutrophil subsets, analysis at the single level should facilitate the dissection of the transcriptional program controlling a given effector function. This way we anticipate that our approach will provide a unique opportunity for a comprehensive characterization of their properties, phenotype and transcriptional regulation in the pre-clinical cancer setting. We anticipate that a clear delineation of the molecular determinants supporting selective anti-tumour activities may provide ground to design novel ways to manipulate myeloid cells in the TME. This will help us to unleash their potential to promote an anti-tumour response leading to tumour regression and ultimately eradication.

# 5. Translation and implication of our findings

In this thesis, we demonstrate that different treatments targeting myeloid subsets can change the TME through the alteration of the tumour-infiltrating macrophage or neutrophil responses. However, and as previously discussed, there are some questions that need to be addressed in order to develop new therapeutic tools to bring manipulation of myeloid cells as one more option of immunotherapy for patients. For instance, strategically it will be important to understand if macrophages and neutrophils perform similar or non-overlapping anti-tumour functions. This information would be critical, along with the nature of the tumour and its content in macrophages and neutrophils to define the best treatment and if combination targeting both macrophages and neutrophils would be beneficial.

Nowadays, the available data regarding the clinical efficacy of immunotherapy in treating cancer shows that using combinatory instead of single agents leads to more effective anti-tumour immune responses. This is probably explained by the fact that human cancers are extremely heterogeneous

and is also able to escape either to immune recognition or killing. This escape can happen by different mechanisms, such as down-regulation of expression of MHC I, expression of inhibitory ligands for T cells, loss of tumour antigen expression or decreased susceptibility to cytotoxicity. Therefore, all these tumour resistance mechanisms might be overcomed by attacking in several fronts.

In 2006, Uno and colleagues first described that for a successful tumour elimination in mice, the activation of multiple steps was required. They used a "trimAb", which is an immunotherapeutic combination of three antibodies that target the TRAIL-R2, CD40 and CD137 (Uno et al., 2006). Further advances and increased knowledge in the onco-immunology field led to propose that four different arms of immunity need to be modulated to enhance the anti-tumour function of the immune system. The first includes the elimination of immune suppression by attenuating suppressor cells with the TME, such as MDSCs, M2 macrophages or even Tregs. Blocking inhibitory receptors in CTLS like CTLA-4, PD-1, Lag-3 or Tim-3 can also be a strategy. Second, induce immunogenic cancer-cell death by using target therapies against the tumour, such as conventional radiotherapy or chemotherapy, use proteasome inhibitors, vaccines, CAR T cells or oncogene inhibitors. Third, a better response can also be obtained through acting on APCs in order to enhance their adjuvanticity. This can be achieved by using immune adjuvants, like GM-CSF or CD40 agonists. Finally, it is also necessary to promote effector T cell activity using agonists against, for instance, ICOS, CD28, OX40, CD27, cytokines, such as IL-2, or even generate antibodies against molecules that are expressed by tumour cells like CD47 or HER2 (Smyth, Ngiow, Ribas, & Teng, 2015).

Indeed, combining anti-cancer therapies has been essential to achieve complete remission in some cancer patients. A key example of this is the effective treatment of melanoma patients by combining antibodies against the checkpoint inhibitors CTLA-4 and PD-1 (Wolchok et al., 2013). Moreover, promising results have also been achieved by combining immunotherapy agents with chemotherapy (Kim, Choi, Kim, Kang, & Kwon, 2008), radiotherapy (Demaria et al., 2005; Newcomb et al., 2010), target therapies (Knight et al., 2013), using anti-angiogenic agents (Hodi et al., 2014; Yasuda et al., 2013) and by partial surgical resections (Kwon et al., 1999).

Therefore, we believe that the best way to improve the effectiveness of cancer immunotherapies will require targeting anti-tumour immune responses at multiple levels, which may be accomplished through synergistic combination of different strategies. Moreover, it is predicted that the majority of these combinations will be built on the PD-1/PD-L1 immune checkpoint blockade (Mahoney, Rennert, & Freeman, 2015; Melero et al., 2015).

Finally, to make the bridge between the combinatory potential and this report, TLR3/CD40 has a better effect on tumour regression than either TLR3 ligand or anti-CD40 alone (data not shown). Moreover, we can also hypothesize that the combination of TLR3/CD40 with a specific immune checkpoint blocker - anti-PD-1 -, could improve the anti-tumour response of CD8+ T cells.

TLRs have been widely studied and some of them are already being used in the clinic or are in clinical trials. For instance, recently, a group of researchers have developed a new cancer vaccine composed by an adjuvant molecule called Diprovocim (TLR2/1 agonist), which synergizes anti-PD-L1 to eliminate B16 melanoma in mice with one hundred percent success. The researchers are now designing further pre-clinical tests for this vaccine, trying to establish how it works in combination with other anti-cancer therapies (Y. Wang et al., 2018).

In conclusion, the work described in this thesis highlights the anti-tumour potential of myeloid cells. We expect that future research will clarify all the biology and mechanisms behind this plasticity and the potential of manipulating these cells in order to improve cancer immunotherapy.
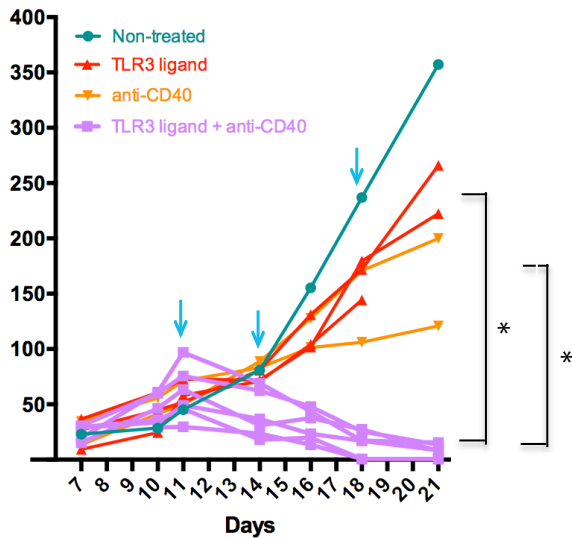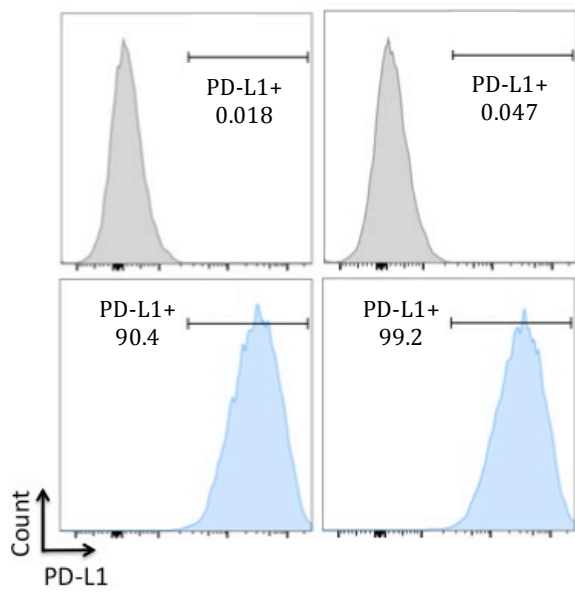
# SUPPLEMENTARY FIGURES
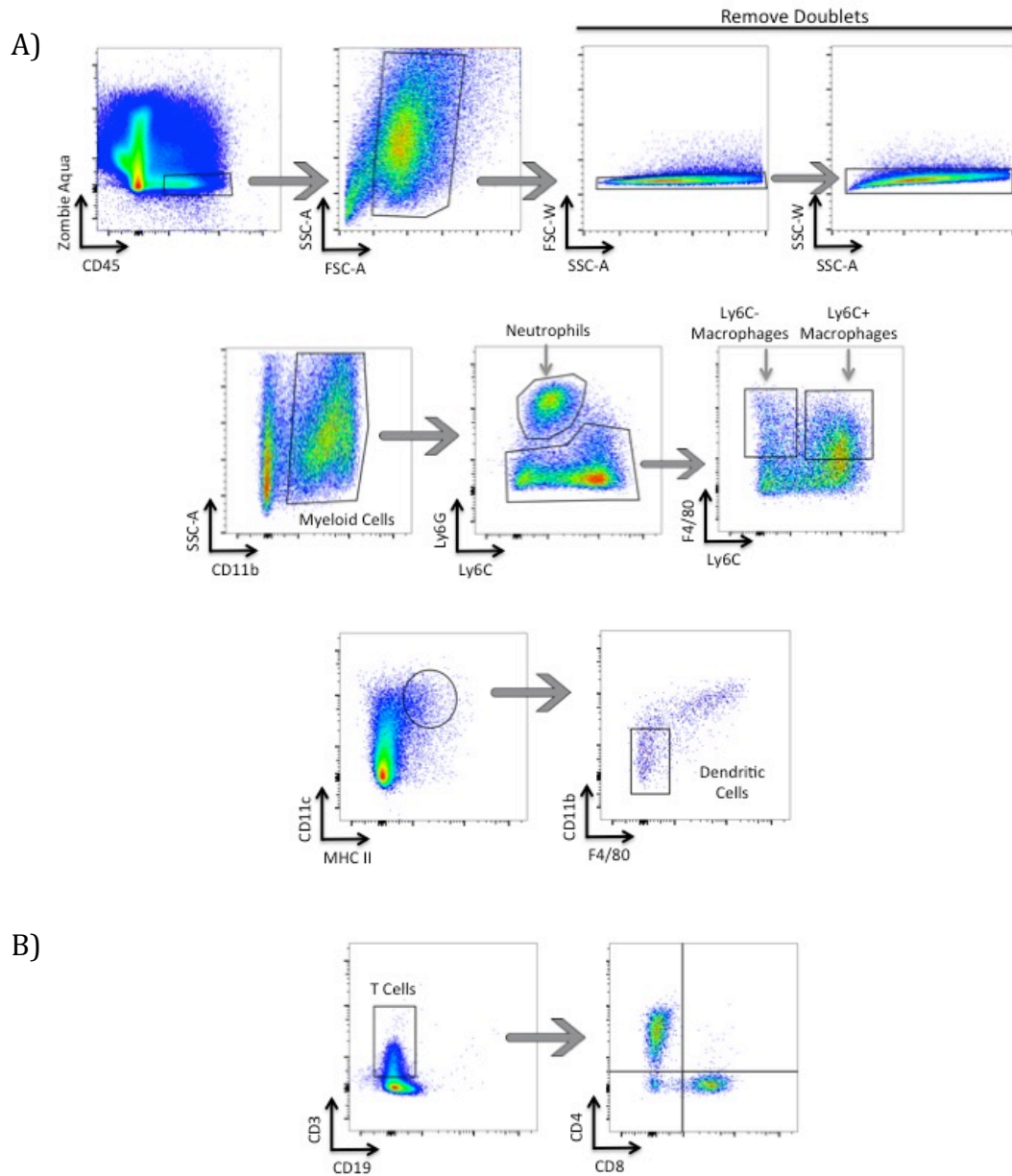


**Supplementary Figure 1:** Flow cytometry gating strategy of **(A)** myeloid and **(B)** lymphocyte populations infiltrating the tumour.
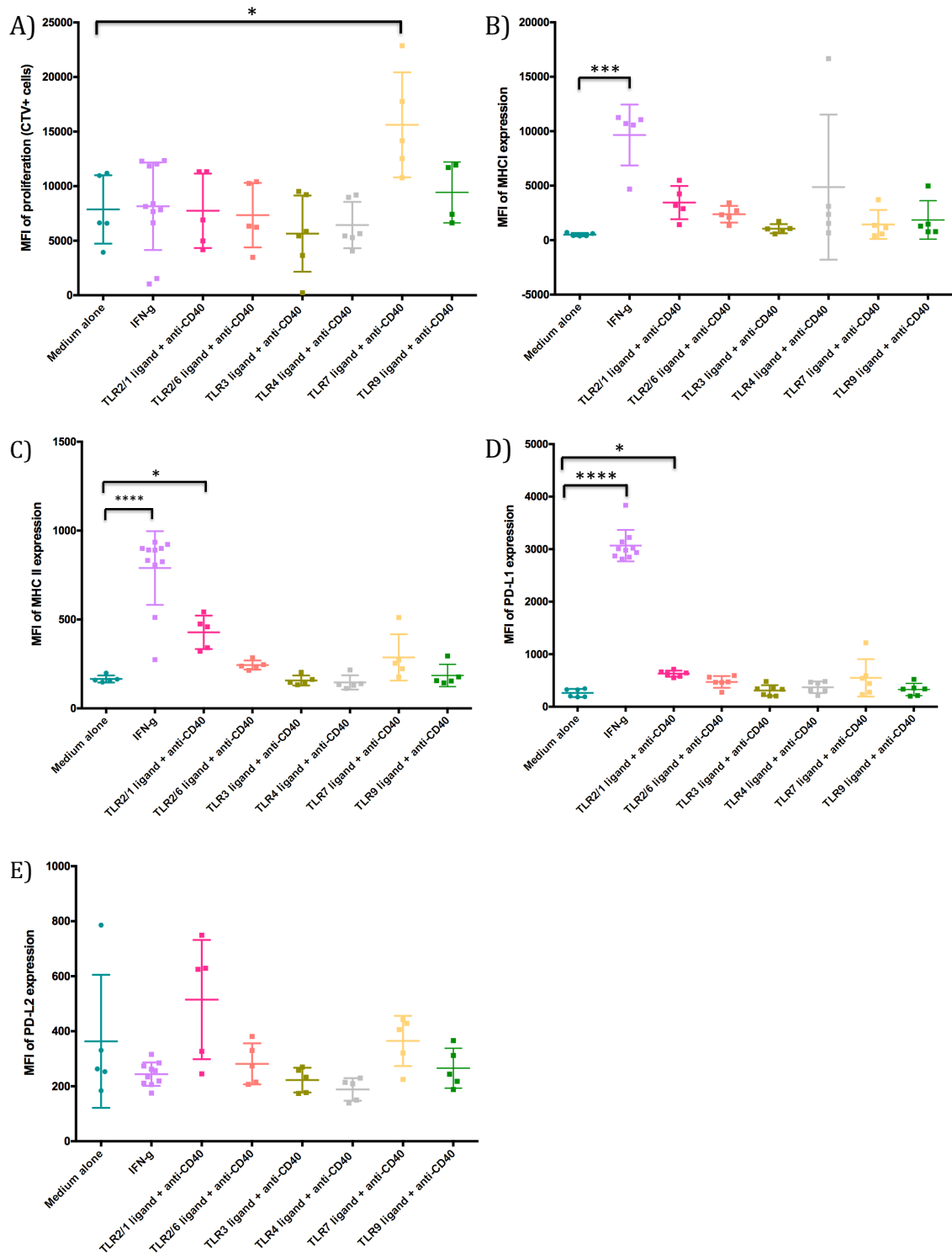
**Supplementary Figure 2:** Graphic representation of **(A)** E0771 tumour cells proliferation and expression of **(B)** MHC I, **(C)** MHC II, **(D)** PD-L1 and **(F)** PD-L2. Statistical analysis was performed using Tukey's multiple compared test.

**Supplementary Figure 3:** Only the combination of TLR3/CD40 leads to tumour complete regression. Non-treated mice (blue circle; n= 1), treated mice with TLR3 ligand (red triangles; n=3), treated mice with anti-CD40 (yellow triangles; n=2) and treated mice with TLR3 ligand plus anti-CD40 (purple squares; n= 6). Mice were treated IT (blue arrows). Statistical analysis was performed using the non-parametric Mann-Whitney test.



**Supplementary Figure 4:** Fluorescence minus one (FMO) control stain of PD-L1 expression.

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Gated on CD3+ T cells

Non-treated    CD8 depletion
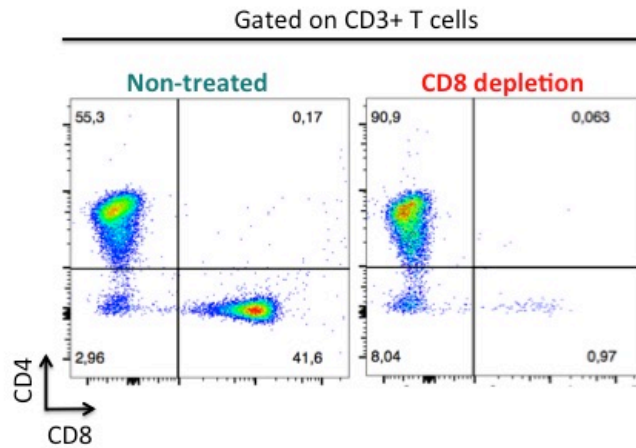
**Supplementary Figure 5:** CD8+ T cell depletion confirmed by blood analysis using FACS on the day of the first treatment.
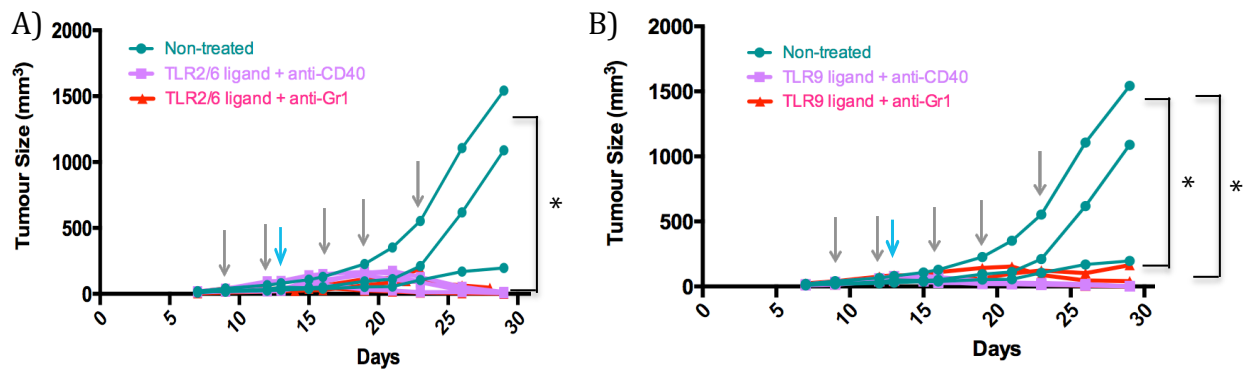


**Supplementary Figure 6:** Neutrophils are dispensable for the elimination of tumour triggered by **(A)** TLR2/6 ligand plus anti-CD40 and **(B)** TLR9 ligand plus anti-CD40.

Non-treated mice (blue circle; n=3), treated mice with TLR ligands plus anti-CD40 (purple squares; n=4) and depleted on neutrophils with anti-Gr1 (red triangles; n=4). Mice were depleted on neutrophils both IV and IP (grey arrows) and injected with TLR ligands plus anti-CD40 IT (blue arrows). Statistical analysis was performed using Tukey's multiple compared test.
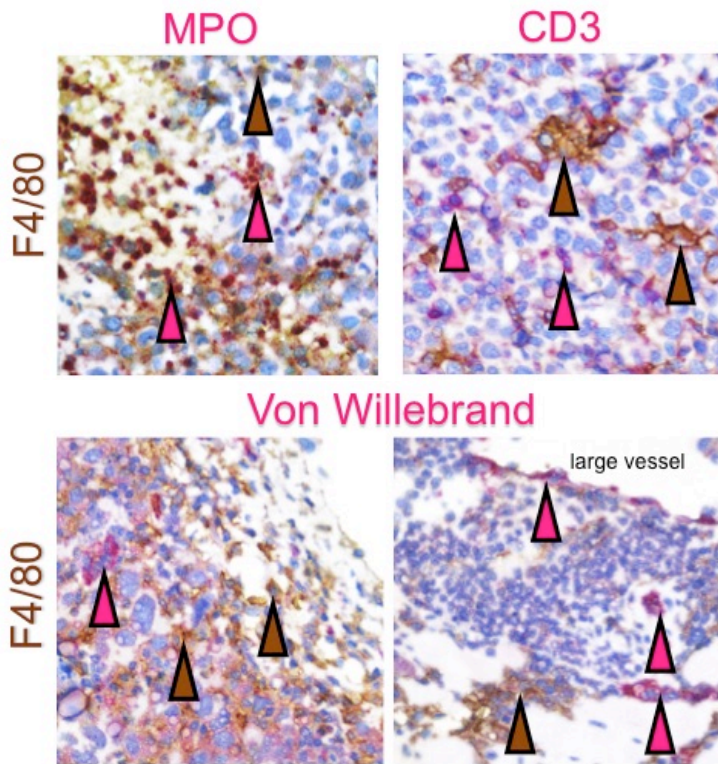
**Supplementary Figure 7:** Immunohistochemistry analysis of non-treated tumours at day 22 after tumour injection. Macrophages (F4/80) are brown and neutrophils (MPO), T (CD3+) and endothelial cells/factor VIII (Von Willebrand) are red. Nucleus of cells are counterstained with haematoxylin (400X).

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