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Targeting antigen-presenting cells: chitosan/poly(γ-glutamic acid) nanoparticles as adjuvants to anticancer therapy

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Article 3

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Article 4

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Review article

F Castro, AP Cardoso, RM Gonçalves, K Serre, MJ Oliveira (2018). Interferon-gamma at the crossroads of tumor immune surveillance or evasion. **Frontiers in Immunology**, 9:847.

Complementary publications in the field:

AP Cardoso, RM Gonçalves, JC Antunes, ML Pinto, AT Pinto, **F Castro**, C Monteiro, MA Barbosa, MJ Oliveira (2015). An interferon-γ-delivery system based on chitosan/poly(γ-glutamic acid) polyelectrolyte complexes modulates macrophage-derived stimulation of cancer cell invasion *in vitro*. **Acta Biomaterialia**, 23,157-171.

GQ Teixeira, CL Pereira, **F Castro**, JR Ferreira, MG-Lázaro, P Aguiar, MA Barbosa, CN-Wilke, RM Goncalves (2016). Anti-inflammatory Chitosan/Poly-ɣ-glutamic acid nanoparticles control inflammation while remodeling extracellular matrix in degenerated intervertebral disc. **Acta Biomaterialia**, 42,168-179.

LM-Teixeira, J Sousa, FW McNab, E Torrado, F Cardoso, H Machado, **F Castro**, V Cardoso, J Gaifem, X Wu, R Appelberg, AG Castro, AO'Garra, M Saraiva (2016). Type I IFN Inhibits Alternative Macrophage Activation during *Mycobacterium tuberculosis* Infection and Leads to Enhanced Protection in the Absence of IFN-γ Signaling. **Journal of Immunology,** 197,4714–4726.

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Porque eu sou do

tamanho do que vejo

E não do tamanho da

Minha altura…

E o que vejo

são os meus sonhos.

Fernando Pessoa

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LIST OF ABBREVIATIONS

A	
A2AR	Adhenosine A2A receptor
ANG2	Angiopoietin-2
APCs	Antigen-presenting cells
ARG1	Arginase 1
ATP	Adenosine triphosphate
C	
CAAs	Cancer-associated adipocytes
CAFs	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
CCL	C-C chemokine ligand
CCR	C-C motif chemokine receptor
Ch	Chitosan
COX2	Ciclo-oxigenase-2
CRC	Colorectal cancer
CSF-1	Colony stimulating factor 1
CSF-1R	Colony-stimulating factor 1 receptor
CTCs	Circulating tumor cells
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte–associated antigen 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
D	
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
E	
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPCs	Endothelial progenitor cells
EPR	Enhanced permeability and retention
F	
FAS L	Fas ligand
FDA	Food and Drug Resistance
FGF	Fibroblast growth factor
FU	Fluorouracil
G	
G-CSF	Granulocytic colony-stimulating factor
G-MDSC	Granulocytic-derived MDSC
H	
HA	Hyaluronic acid
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
HGMB1	High mobility group box-1
HIF	Hypoxia-inducible factor
HSCs	Hematopoietic stem cells
I	
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IGF1-R	Insulin-like growth factor 1 receptor
IL	Interleukin
INOS	Inducible nitric oxide synthase
L	
LPS	Lipopolysaccharide
M	
MCRPC	Metastatic castration-resistant prostate cancer
M-CSF	Macrophage colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MO-MDSC	Monocytic-derived MDSC
MTM	Mammary tissue macrophages
N	
NF-KB	Nuclear factor kappa B
NK	Natural killer cells
NKAR	Natural killer activating receptors
NKG2D	Natural killer receptor group 2 member d
NKIR	Natural killer inhibitory receptors
NKT	Natural killer T cells
NOD	Nucleotide oligomerization domain
NPs	Nanoparticles
P	
PAMPs	Pathogen-associated molecular patterns

PD-1	Programmed cell death 1 receptor
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death ligand 1
PGA	Poly-γ-glutamic acid
PGE2	Prostaglandin E2
PGF	Placental growth factor
PLGA	Poly(lactic-co-glycolic acid)
PRR	Pattern recognition receptors
PyMT	Polyoma middle

R

RT	Radiotherapy

S

SDF	Stromal-derived factor
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription

T

TAAS	Tumor-associated antigens
TAMs	Tumor-associated macrophages
TCR	T cell receptor
TEMs	Tie2-expressing macrophages
TGF-β	Transforming growth factor β
Th	T helper cells
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Treg	T regulatory

V

VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

ABSTRACT

Tumors are not just a heterogenous population of cancer cells but also a variety of resident and recruited host cells, secreted factors and a surrounding extracellular matrix, collectively recognized as the tumor microenvironment (TME). The interaction between cancer cells with their environment ultimately dictates whether the primary tumor is destroyed, persist dormant, or progresses and establishes metastases. The TME can modulate the therapeutic response but also the acquisition of resistance to anticancer therapies, justifying the interest to treat cancer by targeting components of the TME.

Innate immune cells have been assumed as crucial dictators in the tumorigenesis process. They are involved in the initial responses to tissue alterations and can control or prevent tumor initiation and progression, but also facilitate cellular transformation and promote tumor development. This dual behavior is dependent from their own functional and phenotypical plasticity, which makes immune cells attractive targets for the development of novel anticancer therapies. Specifically, macrophages, in response to different stimuli provided by the TME, can adopt different phenotypes in a continuum polarization status with two extreme populations: the pro-inflammatory M1 or the anti-inflammatory M2. Frequently, tumor-associated macrophages exhibit M2 protumor features and dendritic cells (DCs), responsible for antigen presentation and priming of the adaptive immunity, are frequently immature or tolerogenic at the immunosuppressive TME, impairing T cell response. Considering the protumoral role that may be exerted by innate immune cells, several strategies have been explored to reprogram their profile.

Biomaterials have been widely studied, including for vaccine delivery and, importantly, to modulate immune cell functions. Chitosan (Ch) and poly(γ-glutamic acid) (γ-PGA) are non-toxic and biodegradable polymers appealing for biomedical applications, but their potential as immune adjuvants to anticancer therapies had not been exploited. Therefore, the overall aim of this thesis was to develop an injectable system, based on the immunomodulatory properties of Ch and γ-PGA, to modulate immune cells towards an immunostimulatory and antitumoral phenotype. Additionally, the potential of using this system as adjuvant was addressed in combination with radiotherapy or interferon-γ (IFN-γ) therapy in an orthotopic syngeneic metastatic breast tumor model. Our ultimate goal was to evaluate the potential use of these Ch/γ-PGA nanoparticles (NPs) as carriers for IFN-γ, exploiting their impact on immune cell polarization and in impairing immune-mediated cancer cell invasion.

Therefore, the immunomodulatory ability of Ch/γ-PGA NPs was tested on human monocytes-derived macrophages and DCs. The achieved results indicate that Ch/γ-PGA

NPs induced an immunostimulatory DCs profile, with enhanced expression of the co-stimulatory molecules CD40, CD83 and CD86, major histocompatibility complex (MHC) class II and of the pro-inflammatory cytokines IL-6, IL-12/IL-23p40, and TNF-α, eliciting CD4 T cell response. Furthermore, Ch/γ-PGA NPs reprogrammed IL-10-stimulated macrophages, characterized by an immunosuppressive and protumoral phenotype, towards an immunostimulatory profile, reducing the expression of the CD163 scavenger receptor and enhancing the expression of IL-6, IL-12/IL-23p40, and TNF-α, potentiating CD8 T cell response. Additionally, Ch/γ-PGA NPs impaired both macrophages and DCs ability to induce cancer cell invasion.

Posteriorly, the immunomodulatory potential of Ch/γ-PGA NPs in combination with radiotherapy was assessed in the 4T1 breast tumor model. While the effect of Ch/γ-PGA NPs was negligible, the combinatorial treatment potentiated the antitumoral immune response and reduced the systemic immunosuppression, leading to a decrease of both primary tumor growth and lung metastasis formation. In addition, the effect of the combination of Ch/γ-PGA NPs with the immunomodulatory IFN-γ in the 4T1 breast tumor model was also evaluated. This therapeutic strategy decreased myeloid cells in the spleen, specifically the granulocytic myeloid-derived suppressor cells, and attenuated the systemic immunosuppression, leading to a significant decrease of tumor progression.

Lastly, we evaluated the potential of Ch/γ-PGA NPs as vehicles for IFN-γ and assessed its synergism in modulating antigen-presenting cells profile. Although major phenotypic alterations were dependent on Ch/γ-PGA NPs, the incorporation of IFN-γ on NPs potentiated the expression of the co-stimulatory receptors CD40, CD86 and impaired cancer cell invasion.

Taken together, this thesis provides new insights regarding the immunomodulatory potential of Ch/γ-PGA NPs in cancer. Our findings highlight the ability of Ch/γ-PGA NPs to modulate the TME, specifically macrophage and DCs polarization to an immunostimulatory profile, eliciting T cell proliferation/activation, and impairing cancer cell invasion. Their future combination with other therapies could be beneficial for cancer treatment via immune response modulation. In fact, we demonstrated Ch/γ-PGA NPs as adjuvants to radiotherapy and IFN-γ-based therapies likely through the impact on myeloid populations. Moreover, the potential of Ch/γ-PGA NPs as carriers for IFN-γ open new perspectives for their incorporation in nanovaccines formulations, as immunomodulatory and delivery systems. Thus, this PhD work further supports the relevance of targeting the TME, specifically, innate immune cells, by new biomaterials-based therapies for cancer treatment.

RESUMO

Os tumores são constituídos não só por uma população heterogénea de células tumorais, mas também por um conjunto diverso de células residentes e recrutadas, e de fatores solúveis envoltos em matriz extracelular, reconhecidos como microambiente tumoral. A interação estabelecida entre as células tumorais com o seu microambiente determina se o tumor é eliminado, se permanece quiescente, ou se progride e estabelece metástases. O microambiente tumoral pode ainda modelar a resposta do hospedeiro bem como promover a aquisição de resistência aos agentes terapêuticos, reforçando o interesse crescente na modelação do microambiente tumoral.

As células imunes inatas são cruciais no processo de formação dos tumores. Estas células estão envolvidas na resposta inicial à alteração da homeostasia e previnem ou controlam a progressão tumoral. Em tumores mais avançados, podem facilitar a transformação celular e a progressão dos tumores. Este duplo papel é dependente da sua plasticidade funcional e fenotípica, que sugere as células imunes como alvos terapêuticos apelativos ao desenvolvimento de novas terapias para cancro. De entre estas células, os macrófagos em resposta a diferentes estímulos do microambiente tumoral, podem adotar fenótipos distintos num contínuo de perfis de diferenciação entre duas populações extremas: os macrófagos pro-inflamatórios ou M1, e os macrófagos anti-inflamatórios ou M2. Frequentemente, os macrófagos infiltrados nos tumores apresentam um fenótipo similar ao dos macrófagos M2. Por sua vez, células dendríticas (DCs), responsáveis pela apresentação de antigénios e pela ativação da resposta imune adaptativa, apresentam normalmente um perfil imaturo ou anérgico no microambiente tumoral. Considerando os efeitos pro-tumorais que as células imunes inatas podem exercer, várias estratégias têm sido investigadas para reprogramar o seu perfil.

Os biomateriais têm sido amplamente investigados em diferentes áreas, incluindo para formulações de vacinas e, para modelar a função das células imunes. O quitosano (Ch) e o ácido poli-γ-glutâmico (γ-PGA) são polímeros biodegradáveis e não-tóxicos, atrativos para aplicações biomédicas, mas o seu potencial adjuvante para terapias anti-tumorais não foi ainda suficientemente estudado. Portanto, o objetivo geral desta tese foi desenvolver um sistema injetável, nanopartículas (NPs), baseado nas propriedades imunomodeladoras do Ch e do γ-PGA, para modelar o perfil de células imunes para um estado pro-inflamatório e anti-tumoral. Por conseguinte, o potencial adjuvante destas NPs em combinação com a radioterapia (RT) ou com o interferão-γ (IFN-γ) foi avaliado num modelo animal metastático de cancro de mama. Por último, o potencial destas NPs como sistema de entrega do

IFN-γ, bem como o seu impacto na fenótipo de células imunes e na capacidade de estas estimularem a invasão de células tumorais foi explorado.

Portanto, a capacidade imunomodeladora de NPs de Ch/γ-PGA foi avaliada em macrófagos e DCs derivados de monócitos humanos. Os resultados indicam que estas NPs induziram um perfil pro-inflamatório nas DCs, caracterizado pelo aumento da expressão do CD40, CD83 e CD86, do complexo principal de histocompatibilidade (MHC) classe II, da secreção de citocinas pro-inflamatórias, como a IL-12/IL-23p40, IL-6, TNF-α, induzindo uma resposta mediada por células T CD4. Estas NPs reprogramaram também macrófagos estimulados com IL-10, que são caracterizados por exibir um perfil imunossupressor e pro-tumoral, para um perfil pro-inflamatório, reduzindo a expressão do CD163, aumentado a expressão da IL-6, IL-12/IL-23p40 e de TNF-α, e potenciando uma resposta mediada por células T CD8. Adicionalmente, estas NPs diminuíram a capacidade dos macrófagos e das DCs de induzirem a invasão de células tumorais. Posteriormente, o potencial imunomodelador das NPs de Ch/γ-PGA combinadas ou não com RT foi avaliado no modelo de 4T1 de cancro de mama. Enquanto que o efeito das NPs foi negligenciável, a combinação das terapias (NPs + RT) potenciou a resposta imune contra o tumor e reduziu a imunossupressão sistémica, culminando numa diminuição do tumor primário e da formação de metástases no pulmão. Adicionalmente, o efeito da combinação das NPs com o agente imunomodelador IFN-γ foi avaliado no mesmo modelo tumoral. Esta estratégia terapêutica reduziu a acumulação de células mielóides no baço e atenuou a imunossupressão sistémica, resultando numa diminuição da progressão dos tumores. Por último, o potencial das NPs de Ch/γ-PGA como sistemas de libertação de IFN-γ foi avaliado, bem como o seu possível sinergismo na modelação de células imunes. Apesar da maioria das alterações fenotípicas terem sido dependentes das NPs, a incorporação do IFN-γ potenciou a expressão de recetores co-estimuladores, nomeadamente do CD40 e do CD86, e reduziu a invasão de células tumorais.

Como conclusão, esta tese contribui com novos conhecimentos sobre a capacidade imunomodeladora das NPs de Ch/ γ-PGA e do seu potencial terapêutico num contexto de cancro. Os resultados obtidos sublinham o potencial das NPs para modelar o microambiente tumoral, especificamente os macrófagos e as DCs para um perfil pro-inflamatório, com consequente impacto na ativação das células T e na capacidade de induzirem a invasão de células tumorais. A combinação destas NPs com outras terapias poderá ser benéfica para o tratamento do cancro através da modelação da resposta imune. Demonstramos ainda o potencial adjuvante destas NPs para a RT e terapias com IFN-γ, provavelmente através do seu impacto nas populações mielóides. Além disso, estas NPs foram usadas como sistemas de libertação do IFN-γ, sublinhando o seu potencial de serem

usadas em formulações de vacinas para cancro. Em suma, este trabalho suporta ainda a relevância de modelar o microambiente tumoral, nomeadamente, as células imunes inatas, através de novas terapias tendo por base os biomateriais, para o tratamento do cancro.

CHAPTER I

General Introduction

1. Cancer

Cancer is the second leading cause of death worldwide and was responsible for an estimated 9.6 million deaths in 2018 (OMS, 2018). Alarmingly, the number of new cases is predicted to increase by 70% over the next two decades. A key factor contributing to anticancer drug resistance, therapeutic failure and consequently this dramatic outcome, is the intratumor heterogeneity, a consequence of the accumulation of genetic and epigenetic mutations on cancer cells [1].

2. Hallmarks of cancer

Cancer is widely accepted to result from the gradual accumulation of driver gene mutations that successively increase cell aberrant proliferation which upon cells acquiring specific features, will be able to migrate, invade adjacent tissues and eventually metastasize, culminating in malignant disease. The transition from a normal cell to a malignant one is driven by alterations in genes, either by germinal or somatic mutations, generally resulting in oncogenes with dominant gain of function and tumor suppressor genes with loss of function. Such genomic instability enables the generation of mutant cells with selective advantages, as i) self-sufficiency in growth signaling, ii) insensitivity to growth suppressors, iii) evading apoptosis, iv) limitless replicative potential, v) promoting neovascularization, and vi) ability to migrate and invade across basement membranes, which could result in metastatic disease. These tumor cell abilities were appointed by Hanahan and Weinberg (2000) as the main dictators of neoplastic disease outcome [2]. A remarkable progress in cancer research in the last 20 years was achieved through the concept that tumor development presents another dimension of complexity, with a wide repertoire of recruited cells as fibroblasts, endothelial cells, neuroendocrine cells, adipocytes and immune cells, which contribute to the tumor microenvironment establishment. Additionally, a non-cellular compartment has assumed relevance on tumor progression - the extracellular matrix (ECM)- a complex network of macromolecules that support cell survival, proliferation, migration, proteolysis and invasion [3]. All these elements intercommunicate through direct interactions or paracrine signaling with an extensive soluble factors secretion, as growth and pro-angiogenic factors, cytokines, chemokines or matrix metalloproteinases (MMPs), influencing cancer and their neighbor cells behavior, contributing to hallmarks traits acquisition.

Most of solid tumors are densely infiltrated by immune cells. These cells have appeared as drivers of the inflammatory state of premalignant and malignant lesions, where some of them paradoxically act as tumor promoters [4]. In addition, solid tumors have evolved to

avoid immune recognition and to limit the antitumor immunity, thereby evading immunological destruction. Furthermore, the ability of cancer cells to reprogram energy metabolism to efficiently support neoplastic proliferation was also considered a tumor acquiring capability. These advances on cancer biology research were revised in 2011, resulting in a new version of hallmarks of cancer (Fig. 1), where tumors are considered complex organs where each compartment assumes an active role in tumor development [5]. The recognition of the importance of the tumor microenvironment on therapeutic responses and anticancer drug resistance has potentiated new therapeutic strategies, which will be herein explored.

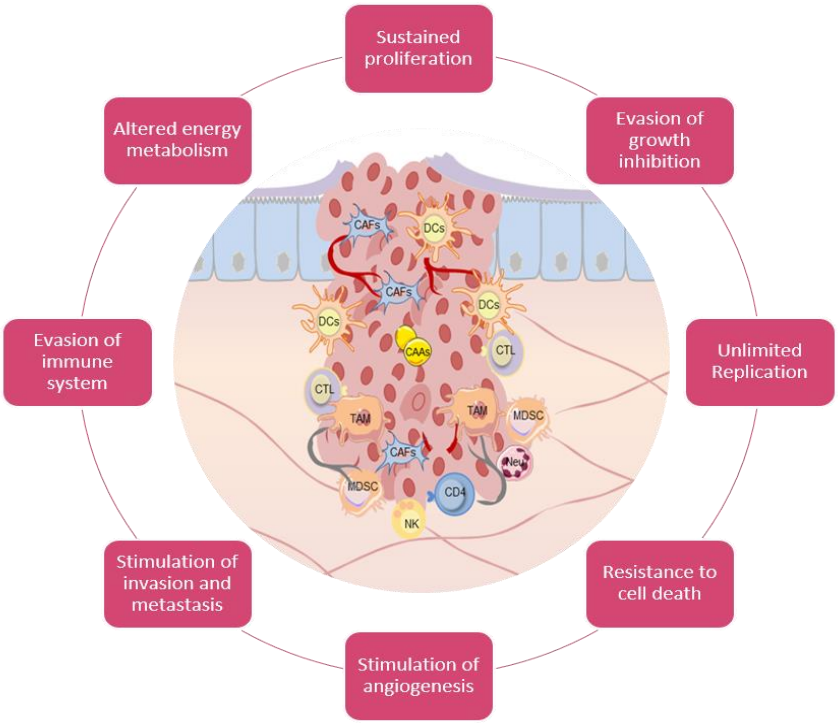

Figure 1. Hallmarks of cancer. These eight biological capabilities are gradually acquired during the multistep development of tumor and are essential for its successful progression. The impact of these hallmarks on tumor microenvironment physiological state (center) determines the tumor outcome. *Adapted from Hanahan and Weinberg, 2011.*

3. The Tumor Microenvironment

Solid tumors are not only a heterogeneous mass of malignant cells but also a variety of resident and infiltrating host cells, secreted factors, blood and lymphatic networks and ECM components, collectively known as the tumor microenvironment (Fig. 2). The interaction between cancer cells with the other elements of their microenvironment influences tumor

progression and, ultimately, dictates whether the cancer cells are eliminated, escape and metastasize or establish dormant metastases.

The structure and composition of the tumor microenvironment is highly variable among different types of cancers and between cancer patients. In addition to cancer cells, tumors are infiltrated by resident and recruited fibroblasts, endothelial cells, pericytes, adipocytes, smooth muscle cells, granulocytes, T and B cells, natural killer (NK) cells, dendritic cells (DCs) and macrophages, which modulate the tumor microenvironment and contribute for the diverse hallmarks of cancer [6]. Their participation will be here briefly discussed.

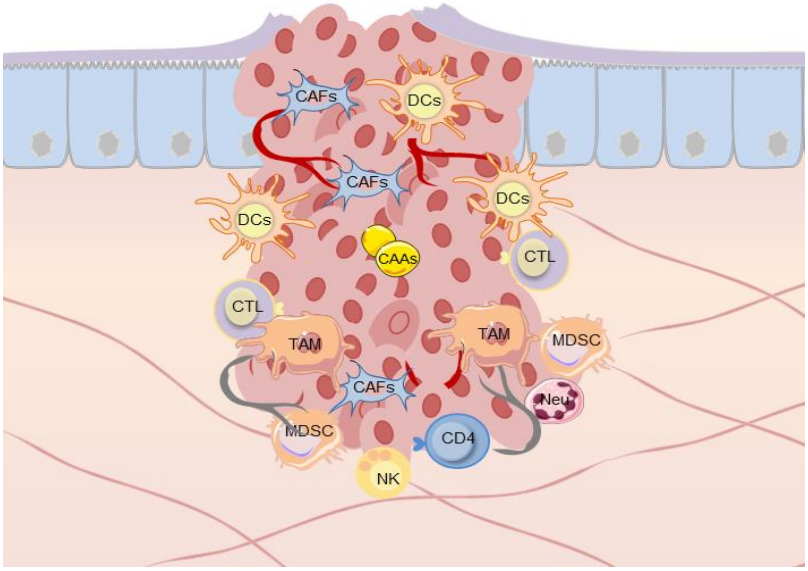

Figure 2. The primary tumor microenvironment. Tumor cells are surrounded by a complex microenvironment composed by stromal cells, namely cancer-associated fibroblasts (CAFs), cancer-associated adipocytes (CAAs), endothelial cells, immune cells, as neutrophils (Neu), dendritic cells (DCs), tumor-associated macrophages (TAMs), CD8 cytotoxic T lymphocytes (CTL), CD4 T lymphocytes (CD4), natural killer (NK) cells, myeloid-derived suppressor cells (MDSC) and by a complex non-cellular compartment – the extracellular matrix (ECM). Blood and lymphatic vessels are also illustrated.

3.1. Tumor cells

Tumor cells result from the accumulation of genetic and epigenetic modifications which, in a given moment, confer to the cell growth advantage, ability to escape to immune cells surveillance, and make them resistant to cell death mechanisms [7, 8]. Although 5 to 10 percent of cancer-related mutations can be inherited, most of them are acquired through errors during normal cell division, a chronic inflammation condition, or certain environmental exposures over individual's lifetime. In fact, these mutational events are frequent and not all mutations acquired by a cell lead to a functional defect. Instead, cells have specific damage surveillance mechanisms, as the DNA damage response signaling, DNA repair

machinery, cell cycle checkpoints, telomere length control and, mechanisms which correct DNA replication errors, avoiding the accumulation of mutations in dividing cells [9]. Nevertheless, when these mechanisms are not enough to correct genetic modifications, aberrant cells are then targeted by the immune system, which usually directs them to cell death, through apoptosis or autophagy induction. However, cancer cells not only downregulate these damage surveillance mechanisms but also subvert them to promote fast evolution and adaptability, where every new mutation acts as driving force of carcinogenesis and metastasis. This process has been proposed as the result of an evolutionary process, where the accumulation of genetic and epigenetic alterations, combined with natural selection mechanisms, face the microenvironment barriers to cancer progression [10, 11].

3.2. Cancer-associated fibroblasts

Non-malignant cells recruited to the tumor have an important role on the support of primary tumor progression, drug resistance and metastasis formation. Among them, fibroblasts are recruited and activated by a gradient of factors, as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β) secreted by cancer and immune cells. When activated, cancer-associated fibroblasts (CAFs) are responsible for the synthesis and remodeling of the ECM, as well as for the secretion of several growth factors, cytokines and MMPs which regulate tumor cell survival, proliferation, immune escape, migration and invasion [12-14]. For example, TGF-β produced by CAFs induces cancer cell epithelial to mesenchymal transition (EMT), sustains an immunosuppressive microenvironment [15, 16] and promotes the activation of ECM-degrading proteases, as Stromelysin-3, also known as MMP3, which cleaves E-cadherin, prompting EMT and cancer cell invasion into adjacent tissues [17]. In addition, TGF-β effects at the tumor microenvironment also includes stimulation of angiogenesis by vascular endothelial growth factor production (VEGF), FGF-2, PDGF-C [18-21] or stromal derived factor-1 (SDF-1), which induce endothelial progenitor cells (EPCs) recruitment and differentiation [22]. Furthermore, CAFs secretome can mediate immune cells recruitment and their regulation by secreting a plethora of cytokines and chemokines, including interleukin-6 (IL-6), IL-4, IL-8, IL-10, tumor necrosis factor-α (TNF-α), TGF-β, C-C motif chemokine ligand 2 (CCL2), CCL5, C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, SDF-1 and prostaglandin E2 (PGE2). However, most of the studies profiling CAFs are from *in vitro* approaches and, therefore, the impact of CAFs on immune modulation *in vivo* remains to be elucidated. Contrarily, the contribution of CAFs, through TGF-β secretion, for the establishment of an immunosuppressive microenvironment has been well described

[23-25]. Importantly, several studies have implicated cancer-associated fibroblasts and their secretome, particularly, TGF-β, hepatocyte growth factor (HGF) and IL-6 in resistance to anticancer drugs [26-28]. All these functional aspects appoint CAFs as attractive targets for anticancer strategies [29].

3.3. Endothelial cells

The relevance of endothelial cells to cancer progression is supported by their involvement in the angiogenesis process, a hallmark of cancer [5]. Tumor-derived factors recruit EPCs, a subtype of stem cells with high proliferative potential, from the bone marrow to the tumor site, where they differentiate into mature endothelial cells, contributing to tumor neovascularization [30]. This process is strictly regulated by the balance between several pro- and anti-angiogenic factors, including FGF and VEGF family members. The imbalance of these factors can activate an "angiogenic switch" [31], resulting in the abnormal vascularization of the tumor. Several cell types have been identified as players of this process, including endothelial cells, vascular smooth muscle cells, stromal and parenchymal cells [32]. This angiogenic switch leads to new vessels formation, which ensure an adequate supply of oxygen, nutrients and growth factors, and elimination of toxic metabolites, but also enables tumor growth and facilitates tumor dissemination and metastasis [33]. The maintenance of endothelial cells on tumors is promoted by self-activation but also by tumor- and stroma-secreted factors, as VEGF and IL-1β, which support endothelial cell survival, proliferation, migration and branching, while interferon-γ (IFN-γ) induces tumor vasculature regression [34]. On their turn, endothelial cells are reported to regulate other cells by producing paracrine signals, as VEGF, SDF-1, PDGF-1, CCL2, and insulin-like growth factor 1 (IGF-1).

Since angiogenesis is essential for tumor growth and dissemination, anti-angiogenic therapies were early proposed as potent weapons against cancer. This awareness appeared in 1971, when Judah Folkman and colleagues hypothesized that tumor growth was angiogenesis dependent [35]. Since then, several efforts have been applied to develop drugs to inhibit VEGF signaling pathway, since one or more VEGF family members are generally overexpressed in most solid cancers. However, several clinical trials showed that these types of drugs, when used as single therapies, do not inhibit angiogenesis for a long time and do not prolong the overall survival of cancer patients [36-38]. In studies using mouse models, the pharmacological inhibition of VEGF decreased primary tumor burden but increased cancer cell invasion and metastasis [39-41]. This can result from a tumor adaptation to anti-angiogenic drugs, by switching VEGF pathway to a different pro-angiogenic pathway, or by recruiting fibroblasts that produce other pro-angiogenic factors,

resulting in resistance to anti-angiogenic drugs [21]. Additionally, the success of tumor response to distinct therapeutic strategies, in particular to radiotherapy, is regulated by endothelial cell function and their ability to induce novel vessels structures [42]. Therefore, the combination of angiogenic inhibitors with conventional therapeutics can potentiate their effects.

3.4. Adipocytes

The role of adipocytes as active players at the tumor microenvironment have gained growing interest by the scientific community [43]. In many tumors, adipocytes are in close contact with the cancer cells [44] and the molecular crosstalk established between these cells can enhance tumor progression [45, 46]. In fact, obesity, a pathological condition associated with an increase and alteration of the adipose tissue function, has been considered as a risk factor for many cancers and for cancer-related mortality [47].

Obesity has been associated with a chronic inflammatory condition, remodeling of the adipose tissue and dysregulation of secreted adipokines, including growth factors, cytokines, chemokines and hormones, as leptin and adiponectin. These adipocyte-secreted factors can act locally or systemically, playing an important role in tumor growth, invasion, angiogenesis [48], metastatic dissemination [45, 49], immune escape, and resistance to treatments [50, 51]. In addition, cancer cells can induce adipocytes phenotypic and functional modifications, being recognized as cancer-associated adipocytes (CAAs) [52]. Several studies have shown that mature adipocytes in contact with cancer cells completely lose their lipid content and exhibit a fibroblast-like morphology, contributing for tumor progression and metastasis [53, 54]. Additionally, co-cultures of cancer cells with adipocytes revealed that these cells may change their secretome profile, characterized by an upregulation of osteopontin, MMP9, TNF-α, IL-6 and IL-1-β [54, 55]. The adipocyte-secreted hormones have been also involved in tumor growth. Importantly, leptin secretion by adipocytes was upregulated in obesity patients and different studies have pointing it as a tumor growth promoter [56, 57] by inducing the signal transducer and activation of transcription factor 3 (STAT3) [58], and by promoting proliferation and angiogenic differentiation of endothelial cells expressing the leptin receptor [59]. Contrarily, adiponectin secretion is downregulated in obesity condition [60], and its role on inhibiting tumor growth is through the binding of growth factors, as PDGF-BB, FGF and heparin-binding EGF-like growth factor (HB-EGF), inhibiting their interaction with their own receptor [61]. Additionally, adiponectin reduced tumorigenesis in several *in vivo* studies [62, 63] and its deficiency promoted tumor growth [64, 65]. Interestingly, a positive correlation between a high leptin:adiponectin ratio and an increased risk of several cancers was established [66, 67].

In addition, the production of pro-inflammatory cytokines by adipocytes, namely IL-6, has been shown to promote tumor growth in different types of cancer [54, 68]. For example, IL-6 secreted by adipocytes signaling through STAT3 induced EMT phenotype in breast cancer cells [69]. However, the antitumor effect of IL-6 blocking was only observed in high fat diet-induced obese mice, but not in normal ones, which suggests that the inflammatory state in obese conditions can influence the tumor outcome [46]. Moreover, adipocytes can modify the immune microenvironment by recruiting pro-inflammatory macrophages through the CCL2/IL-1β/CXCL12 axis, creating a permissive niche for cancer initiation [70]. All these findings support the interest on adipocytes and macrophages crosstalk in cancer, which can be crucial for the prevention and treatment of obesity-associated cancers.

3.5. Immune cells

The regular presence of leukocytes within tumors, observed as early as 1863 by Rudolf Virchow, provided the first indication of a possible link between chronic inflammation and cancer [71]. Indeed, solid tumors are infiltrated by different immune cell populations which establish dynamic interactions within the tumor microenvironment and shape disease progression [4]. These immune cells are responsible for tissue homeostasis maintenance, through the recognition and elimination of foreign invaders, and for the removal of damaged or transformed cells which escape DNA repair or cell death mechanisms. However, they frequently appear to be modulated by cancer cells, becoming tolerogenic and, consequently, dampening antitumor immune responses and promoting cancer-related activities [72].

The tumor immune infiltration encompasses numerous cellular phenotypes, including myeloid cells, namely macrophages, DCs, mast cells, granulocytes (neutrophils, eosinophils and basophils), myeloid-derived suppressor cells (MDSCs), innate lymphoid cells, as γσ T cells, NK cells and NKT cells, which mediate a primary innate immune response – rapid and antigen unspecific [73]. Instead B and T cells, which recognize specific nonself antigens presented by antigen-presenting cells (APCs), enable the adaptive immune response [74]. The immune infiltrate composition, their maturation and spatial distribution are variable among different cancers but also between cancer patients, with a relevant impact on the clinical outcome [75, 76]. Since the tumor associated-immune cells phenotype, function and abundance seem to be crucial regulators of cancer progression and therapy response, understanding how these cells can be reprogramed or targeted have been extensively studied. Therefore, the role of key immune cell players on the tumor microenvironment will be here briefly addressed.

3.5.1. Natural killer cells

NK cells are granular lymphocytes endowed with ability to recognize and kill pathogens and malignant cells. They are recruited to the tumor site following a gradient of chemokines, namely CXCL9, CXCL10 and CXCL11 [77]. Their activation is dictated through a balance between negative signals provided by inhibitory receptors (NKIR), upon interaction with MHC I molecules, and positive signals conducted by a diversity of activating receptors (NKAR) [78]. Between them, lectin-like receptor natural-killer receptor group 2 member D (NKG2D), a potent activating receptor constitutively expressed on the surface of NK cells, but also on NKT cells, γδ T cells, and some CD8 and CD4 T cell subtypes, recognize specific alarming signals in a cancer or infection-dependent context [79].

In cancer cells, genomic-induced stress increases their sensitivity to NK cell-mediated lysis by the induction of NKG2D ligand expression [80, 81]. Upon activation, NK cells have the ability to destroy damaged or transformed cells, typically those that do not express MHC I molecule on their surface [82]. They can induce tumoricidal effects via perforin and granzymes secretion [83] or through the engagement of the cell death surface receptor Fas by Fas ligand (FasL), mediated by caspase activation [83, 84] or by IFN-γ secretion [85]. Furthermore, NK cells pursuit the capacity to regulate DC-mediated antitumor immunity, by inducing DCs to activate T helper 1 or CD8 T cell responses [86, 87], by providing antigenic material for DCs presentation [88], and by killing immature DCs [89]. This crosstalk between NK cells and DCs also potentiates NK cell functions [90-92]. However, cancer cells can develop strategies to subvert NK cell cytotoxic functions by several mechanisms, including through TGF-β production [93], downregulation of NKAR ligands [94, 95], upregulation of NKIR ligands as programmed death-ligand 1 (PD-L1), upregulation of MHC class I [96-98], and downregulation of Fas [99].

Due to their pro-inflammatory and tumoricidal activities, NK cells cytotoxicity has been correlated with good prognosis in multiple cohorts of cancer patients with or at risk of metastatic disease [100-103], and an inverse correlation between high amounts of tumor-infiltrating NK cells and the presence of metastases has been verified [104, 105]. In fact, several preclinical studies reported that NK cells mediate robust antimetastatic effects (reviewed in [106]), renewing the interest on the possibility of harnessing NK cell functions against metastatic disease [107].

3.5.2. Neutrophils

Neutrophils are the most abundant immune cell population, representing at least 50% of all leukocytes [108]. Neutrophils arise from lymphoid-primed multipotent progenitors, which are

derived from hematopoietic stem cells, in a process named granulopoiesis, where the granulocyte-colony stimulating factor (G-CSF) is the main regulator of neutrophil generation and differentiation [109]. These cells are the first responders to infection and acute tissue injury, being specialized in phagocytosis and intracellular killing of pathogens, production of reactive oxygen species, release of granules with antimicrobial peptides, and in the formation of neutrophil extracellular traps [110]. Neutrophils are not just “killers” but also orchestrates innate and adaptive immune responses by secreting cytokines, chemokines and by inducing antigen presentation. These cells can express lower levels of MHC class II and co-stimulatory molecules, inducing a T helper 1 or T helper 17 differentiation [111].

Primary tumors can activate “emergency granulopoiesis” in the bone marrow, a process that overtakes the stead and continuously stimulate the release and the recruitment of mature neutrophils, but also of undifferentiated cells [112]. In tumor-bearing mice and in some human cancers, neutrophils production can also occur in the spleen [113-115]. Indeed, patients with different types of cancer frequently present increased number of neutrophils in circulation [116] and at the tumor microenvironment, generally associated with poor prognosis [117-119], which suggests that these cells might have significant protumor activities. However, the role of neutrophils in cancer is still controversial, since contrasting roles during cancer progression have been described. In fact, it is known that neutrophils display functional plasticity and, depending of soluble mediators released at the tumor microenvironment, they can polarize into different activation states - an antitumoral (N1) or a protumoral (N2) profile [120]. Thus, some studies have suggested that neutrophils can exhibit an antitumor role by activating T cell responses against tumor cells [121, 122]. On the other hand, MMP9-secreting neutrophils are critical to facilitate angiogenesis and tumor intravasation [123]. Neutrophils can also produce high amounts of reactive oxygen species, potentiating cell mutagenesis, and consequently, carcinogenesis [124]. Other researchers demonstrated that neutrophil-derived oncostatin M induce VEGF production by breast cancer cells and increased their invasive capacity [125]. Moreover, in a pancreatic model neutrophil-derived MMP9 increases VEGF, while a transient depletion of neutrophils impaired VEGF-induced angiogenic switch [126].

The role of neutrophils on metastasis has been confirmed by more recent studies. Spicer *et al.* demonstrated that neutrophils promote liver metastasis by facilitating adhesion of already-circulating tumor cells (CTCs) to the liver parenchyma through Mac-1/ICAM-1 interaction, and neutrophils depletion before cancer cell inoculation resulted in fewer metastases [127]. Further, CTCs were found to be associated with neutrophils in human breast cancer. Additionally, the transcriptome of CTCs combined with neutrophils presented several genes differentially upregulated which potentiate metastasis, and patients with at

least one CTCs-neutrophil cluster had worse progression-free survival in comparison with patients with five or more CTCs alone (previously defined as the threshold for adverse outcome) [128]. These results corroborate with the previous studies proposing that neutrophils prime the pre-metastatic niche [129-132]. Additionally, the inhibition of myeloid cells recruitment through the blocking of C-X-C chemokine receptor 2 (CXCR2) in a breast carcinoma model enhances the efficacy of chemotherapy against primary tumors and metastasis [133], suggesting that targeting neutrophil recruitment could lead to more efficient therapeutic strategies in cancer.

3.5.3. Myeloid-derived suppressor cells

MDSCs are immature myeloid cells at different stages of differentiation. They include two main subsets, the monocytic (Mo-MDSC) and the granulocytic (G-MDSC) subgroups, which exploit different mechanisms of immune suppression [134, 135]. MDSCs are recruited to the tumor microenvironment in response to a chemokine gradient, mainly CCL2, CCL15 and SDF-1 [136, 137], where they exert potent immunosuppressive activities, supporting tumor progression (reviewed in [138]). They are described to inhibit T cell functions, through the production of reactive oxygen species [139], and to inhibit their recruitment at the lymphoid tissues [140]. MDSCs upregulate the expression of several factors associated with immunosuppressive activities, including arginase 1 (ARG1), inducible nitric oxidase synthase (iNOS), TGF-β, IL-10, cyclooxygenase-2 (COX2), indoleamine 2,3-dioxygenase (IDO) and PD-L1 [141]. These factors modulate immune cells profile, promote the activation and proliferation of the immunosuppressive T regulatory (Treg) cells [142], the acquisition of an immunosuppressive macrophage profile (M2-like) [143] and the inhibition of DCs differentiation [144], prompting the acquisition of an immunosuppressive tumor microenvironment. MDSCs can also promote tumor progression by affecting angiogenesis and tumor dissemination through the secretion of pro-angiogenic factors, MMPs and by inducing EMT [145-147]. Considering the MDSCs immunosuppressive and protumoral role, several preclinical and clinical studies were performed to target MDSCs recruitment or their activities, resulting in tumor growth inhibition and improved survival (reviewed in [148]).

3.5.4. Dendritic cells

DCs are differentiated myeloid cells and are the most efficient APCs. They are specialized in antigens processing, converting them into peptides that can be presented to naïve T cells in MHC class I and II context, eliciting the adaptive immunity [149]. DCs also have an important role in initiating immunological tolerance, memory, and in the regulation of T cell-mediated immune responses [150, 151]. DCs family is heterogeneous, comprise several

subsets of cells with specific phenotype and function, including the most well described conventional DCs and plasmacytoid DCs [152, 153].

Generally, DCs work together with macrophages as sentinels of the immune system. They express several receptors, including pattern recognition receptors (PRRs) that recognize pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) in the foreign stimuli. Malignant cells can undergo cell death by immunological recognition by macrophages and neutrophils, by nutrients, or by oxygen deprivation, resulting in the delivery of danger signals, as the secretion of adenosine triphophosphate (ATP), heat shock proteins and the release of the chromatin-binding protein high-mobility group box 1 (HMGB1) (reviewed in [154]). Immunological signals provided by damaged or dying tumor cells prompt antigen recognition by DCs and their functional maturation. This state leads to: i) the upregulation of MHC class II and of costimulatory molecules, such as CD40, CD83, CD86, at the cell surface; ii) the upregulation of CCR7 that enable DCs migration through the axis CCR7-CCL19/21 to the lymph nodes; and iii) provides the capacity to secret pro-inflammatory cytokines (TNF-α, IL-12, IL-6, IL-1α and IL-8), promoting the differentiation of naïve T cells into effector T cells [155, 156], as well as the promotion of NK cell functions [157] and B cells differentiation and activation [158].

The relation between tumor-infiltrated DCs and patient clinical outcome is not well understood, with some reports associating the presence of DCs with good prognosis and others with poor prognosis (reviewed in [159]), reflecting the complexity of their phenotype and of their activation status [160]. Recently, it was also demonstrated that high density of mature DCs at tertiary lymphoid structures correlate with T helper 1 and CD8 T cell infiltration and with improved survival of lung cancer patients [161]. Frequently, tumor-infiltrated DCs are unable to stimulate an immune response to tumor-associated antigens. In fact, the immunosuppressive and hypoxic tumor microenvironment impairs DCs differentiation, activation and function [162], resulting in the deficient formation of antitumor immune response or in the development of DC-mediated tolerance. In order to subvert DCs tolerance, several therapeutic strategies have exploited the immunoregulatory properties of DCs for cancer treatment. The main goal of cancer vaccines strategies involving DCs is to stimulate antigen-specific CD8 T cells, which recognize and eliminate tumor cells in an antigen-specific way. This strategy includes the administration of autologous DCs treated *ex vivo* with tumor antigens and a cocktail of immunomodulators agents (pro-inflammatory cytokines, CD40L and toll-like receptors (TLRs) agonists) [163]. Despite their favorable safety profiles, clinical responses have been quite disappointing, with classic objective tumor response rates hardly exceeding 15% [164]. Recently, advanced melanoma patients treated with DCs co-electroporated with TriMix-mRNA (CD40L-, CD70-, and caTLR4-

encoding mRNA) and mRNA encoding one of the four melanoma-associated antigens (MAGE-A3, MAGE-C2, tyrosinase, or gp100) fused to an HLA class II-targeting signal (DC-LAMP) (also called TriMixDC-MEL), when in combination with ipilimumab, achieved an encouraging overall response rate of 38% [165]. Therefore, the current challenges to improve the efficacy of DCs-based therapies include the optimization of DCs maturation protocols, combined with approaches that stunned immune evasive mechanisms promoted by the tumor microenvironment.

3.5.5. Lymphocytes

Lymphocytes are the cellular effectors (T and B cells) of the adaptive immunity that, oppositely to the innate response, creates an immunological memory against specific antigens. After antigen recognition, lymphocytes orchestrate a robust response to eliminate the pathogen, and in case of re-exposure, they can protect the host from it. T cells are divided in αβ T and γσ T cells, accordingly to the chains that constitute their T cell receptor (TCR). Naïve αβ T cells are classically divided into either $CD8^+$ cytotoxic T cells or $CD4^+$ T helper (Th) cells that recognize antigens presented by MHC class I or II, respectively. After primed in the lymph nodes, $CD4^+$ T cells orchestrate several immune responses through secreting cytokines and chemokines that activate or recruit target cells [166].

$CD4^+$ T cells further activate macrophage pro-inflammatory and antimicrobial activities, recruit granulocytes to the inflammatory site, potentiate the production of antibodies by B cells and support primary and memory $CD8^+$ T cell responses [167, 168]. Depending on the nature of the antigen stimulation signal received from APCs, $CD4^+$ T cells can further differentiate into different effector T helper or regulatory T cell phenotype. Several $CD4^+$ helper T cells phenotypes are described, being the most studied Th1, producing IFN-γ, IL-2 and TNF-α; Th2, producing IL-4, IL-10 and IL-13; and Th17, secreting IL-17, IL-6, IL-21 and IL-22. These cells display specific transcriptional programs, reflecting their differential functions. In the cancer context, all these phenotypes are reported to be involved in tumor immunity (reviewed in [169]). The greater antitumor actions of Th1 cells are due to their high production of IFN-γ, as well as the chemokines that potentiate the activation and expansion of $CD8^+$ T cells, further recruiting macrophages and NK cells to the tumor site, which together concert an antitumor immune response [170-173]. The Th2 cells contribution to antitumor immunity is still contradictory and seems to be context dependent [174, 175]. Nevertheless, it is consensual that Th2 cells produce cytokines/chemokines which promote a tumor immunosuppressive microenvironment. Additionally, patients with bladder, gastric and colorectal cancer (CRC) showed lower proportions of Th1 cells ($IFN\text{-}\gamma^+$ $IL\text{-}2^+$) while

exhibiting higher proportions of Th2 cells (IL-4$^+$ IL-6$^+$ IL-10$^+$), in comparison with healthy controls [176, 177].

Interestingly, Th17 cells produce beyond IL-17, various chemokines including CCL2, CCL7, CXCL1 and CCL20 as well as MMPs which promotes the inflammatory process. High IL-17 levels have been associated with chronic immune responses [178], an essential conditional for the promotion of carcinogenesis [179]. Additionally, Th17-producing IL-6 activates STAT3, a critical transcription factor for carcinogenesis promotion [180], which further activates Th17 differentiation, amplifying IL-6 in the environment, and the survival NF-KB pathway. However, the role of IL-17 in tumor development is controversial [181, 182]. Hepatocellular carcinoma patients with high infiltrated IL-17-producing cells had a poor survival [183]. Intratumoral IL-17 induced the expression of angiogenic chemokines, as VEGF by tumor cells, promoting angiogenesis [184, 185]. In addition, IL-17 promoted tumor development in several tumor models, by inhibiting CD8 T cells while increasing MDSC infiltration in the tumor [186]. Several strategies to inhibit the IL-17 pathway have been established for autoimmune diseases and are being currently tested in the cancer context [187].

A population of CD4$^+$ Treg cells are responsible for inhibiting aberrant self-reactive lymphocytes. They are described to have antitumor activities, and patients with elevated number of Treg cells intratumorally have often poor prognosis [188]. Studies showed that the depletion of Treg cells enhance antitumor CD8 T cell response [189]. Recent advances in cancer immunotherapy showed that patients treated with anti-cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) therapy had low levels of Treg cell number in tumor tissues [190], indicating that Treg cells targeting, direct or indirectly, is an encouraging strategy to improve anticancer treatments.

CD8$^+$ T cells, often called cytotoxic T lymphocytes (CTLs), assume a preponderant role in antitumor immunity. After activation in MHC class I context, they orchestrate a potent tumoricidal program [191]. CD8$^+$ T cells produce TNF-α and IFN-γ, which have antitumor properties, and release cytotoxic granules containing perforins and granzymes, resulting in target cell apoptosis. Furthermore, they can induce tumor cell destruction through the activation of the FasL/Fas pathway [192].

Although $\gamma\sigma$ T and NKT cells are present in lower numbers, they are important to bridge innate and adaptive immune systems. They combine the expression of TCR and recognize antigens independently of the presence of APCs. In particular, NKT cells express cytotoxic receptors at the surface and both cell types can have cytolytic functions, leading to tumor cells eradication [193, 194].

B cells secrete specific antibodies against tumor antigens which opsonize the target cell or lead to antigen processing and presentation by APCs, activate the complement cascade and contribute to NK cell mediated tumor killing [195]. Although antibodies against tumor antigens have been frequently detected in the serum of cancer patients [196], the role of humoral immunity in cancer remains controversial, probably due to the existence of different B cell phenotypes. Other reasons could be that many of the antibodies in cancer patients are directed to self-antigens, expressed on cancer and in non-malignant cells [197]. Furthermore, they are also described to limit antitumor immunity by inhibiting Th1 and $CD8^+$ T cell responses while potentiate Th2 cell protumor responses (reviewed in [198]).

Overall, high number of lymphocytes, namely CD3, CD8 as well as a high CD8/FoxP3 ratio have been described to have a positive effect on patient survival in several types of solid cancers [199]. Fridman *et al.* also showed that $CD8^+$, $CD45RO^+$ (memory T cell) and Th1 T cells are both found to be a positive prognostic factor in the vast majority of the studies [200]. T cells are being currently used in chimeric antigen receptor (CAR) T cell therapy [201] to potentiate their effects at the tumor site.

3.5.6. Macrophages

3.5.6.1 Origin and functions

Macrophages are distributed in nearly all tissues within the body, exhibiting distinct anatomical and functional diversities, and ability to widely adapt to the different environments. They play key roles in development, angiogenesis, immunity, host defense and tissue repair, contributing to both homeostasis and disease [202]. Their origin was not consensual for many years. Currently, it is known that macrophages within adult tissue can have three major sources, derived from the embryonic yolk sac, the fetal liver or from bone-marrow-derived circulating monocytes. During embryogenesis, erythromyeloid progenitors, derived from the embryonic yolk sac, enter in the bloodstream and give rise to tissue-resident macrophages distributed throughout tissues, namely fetal liver, brain, bone marrow, kidney, spleen, lung and skin. Hematopoietic stem cells (HSCs) colonize posteriorly the fetal liver, where they expand, colonize the bone marrow, and differentiate into fetal-derived monocytes that enrich the macrophage population in the previously stated organs, persisting in the adult as resident and self-renewal, independently of the pool of circulatory monocytes. HSCs in the bone marrow are the source of blood circulating monocytes which are recruited with high turnover in inflammation or disease context [203, 204].

Macrophages, professional phagocytes from the innate immune system, are constantly surveilling throughout the tissues, being responsible for the recognition and triggering of host defense against foreign stimuli. As sentinel cells, they are prepared with a plethora of damage signals sensors PRRs, including TLRs, diverse C-type lectins or cytosolic sensors, such as nucleotide-binding oligomerization domain (NOD)-like receptors, to recognize danger signals from pathogens or tissue damage [205]. Upon a microbial challenge, macrophages sense certain structures present in many pathogens, known as PAMPs, though their PRRs, conferring a degree of specificity. For example, TLR4 recognizes liposaccharides (LPS) on the surface of Gram-negative bacteria. After this engagement, macrophages can directly eliminate the pathogens by phagocytosis and lysosomal activation. They can activate a potent pro-inflammatory and oxidative program, secreting high levels of TNF-α, IL-1, IL-12 and IL-23, reactive oxygen species, leading to the recruitment of immune cells, as neutrophils, additional monocytes, DCs and, if not resolved by innate compartment, T cells are also recruited, which ultimately lead to pathogen control and/or elimination. On its turn, the resolution of inflammation initiates with chemokines depletion, either by MMPs cleavage, rendering them non-functional, or by sequestering by decoy receptors, neutrophils entering in apoptosis and secreting factors that inhibit the sustained neutrophil infiltration. Macrophages uptake apoptotic neutrophils, switching their profile towards a resolution-phase macrophage, characterized by the production of anti-inflammatory cytokines as TGF-β and IL-10, VEGF and lipoxin A4, contributing to tissue regeneration and reestablishment of its functionality (reviewed in [206]). More recently, Treg cells [207] and MDSCs [208] have assumed also a role in the resolution-phase of inflammation. This differential response reflects the ability of macrophages to adapt and respond to different immunological contexts, highlighting the importance of their response being tightly regulated in time and space, to avoid chronic inflammation and consequently, tissue injury.

3.5.6.2 Macrophage classification

Macrophages are likely the most functionally diverse and plastic cells of the immune system. Depending on the diversity of the external cues that macrophages are exposed to, they constantly adjust their phenotype and behavior to maintain tissue homeostasis. The first attempts to classify activated macrophages divided them in two groups: the classical activation and the alternative activation for CSF-1/IFN-γ [209] or IL-4/IL-13 [210, 211] stimuli, respectively. Later, Mills and colleagues found that macrophages activated in mouse strains with Th1 and Th2 backgrounds respond to IFN-γ or LPS in a different way, proposing a M1 and M2 classification, which mimics the Th1-Th2 dichotomy [212].

However, recent advances in the field showed that macrophage phenotypic and functional heterogeneity is more complex than initially considered. In fact, different environmental factors, as chemokines, cytokines, growth factors, PAMPs, DAMPs, and hormones differentially regulate macrophage activation and response, resulting in distinct phenotypes [213]. More interestingly, these macrophage phenotypes are only sustained in the presence of the stimuli, and under their absence, the phenotype reverses, make them appealing targets of immunomodulatory therapies. Mantovani and colleagues proposed a new classification system, dividing M2 macrophages in three subtypes (M2a, M2b, M2c) depending the stimuli that they respond to. All these phenotypes have specific cell surface markers and their specific secretome and, consequently, different functions. M2a are induced by IL-4 and IL-13 and they are involved in Th2 inflammatory response and parasite killing. M2b are prompted by the exposure to immune complexes and agonists of TLRs or of the IL-1 receptor (IL-1R), being responsible for regulation of inflammation and immunity. On their turn, M2c are mainly induced by IL-10 and involved in matrix deposition, tissue remodeling and regulate the inflammation [214]. More recently, a M2d phenotype induced by adenosine A2A receptor (A2AR) and TLR agonists was described. M2d expresses high levels of VEGF, IL-10 and are involved in angiogenesis promotion, but further studies are required [215]. On the other hand, M1 macrophages are induced by TNF-α, LPS or through the combination of IFN-γ with LPS, and are associated with the Th1 response, pathogens killing and antitumor properties. This classification comprises a continuum of diverse functional states where M1 and M2 are the extremes of this spectrum (Fig. 3) [214]. Considering that macrophages can share characteristics from different phenotypes, Mosser and Edwards suggested an alternative macrophage grouping based on three different homeostatic activities – host defense, wound healing and immune regulation, represented in a colored wheel. It brings together, classically activated macrophages, wound-healing macrophages and regulatory macrophages, respectively [216]. Recently, Peter Murray and colleagues proposed a nomenclature and experimental guidelines to incentive the scientific community to adopt the same terminology and experimental standards in *in vitro* procedures [217]. *In vivo*, it was proposed that macrophages with mixed phenotypes can coexist in the same environment and are affected by dynamic changes during disease evolution. In fact, they are implicated in the development of several diseases associated with chronic inflammation, as arthritis, atherosclerosis, diabetes, asthma and cancer [218].

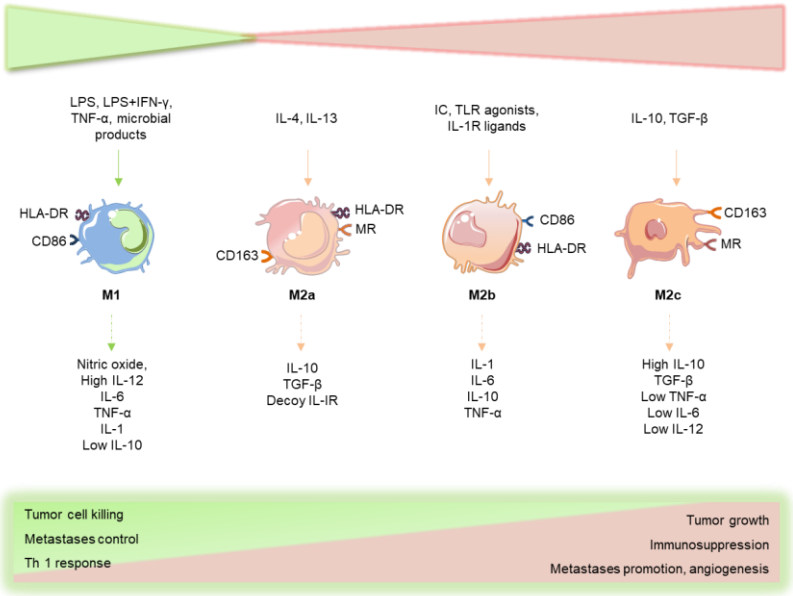

Figure 3. Stimuli and selected functional properties of different macrophage phenotypes. Macrophages polarize and acquire different functional properties in response to distinct environment-derived signals. Macrophage exposition to LPS, IFN-γ, TNF-α and microbial produts drives M1 polarization, potentiating cytotoxic and antitumoral properties, while M2 macrophages are frequently more prone to immunoregulatory and protumoral activities. Specifically, M2a macrophages, induced by IL-4 and IL-13, and M2b macrophages, polarized by the combination of exposure to immune complexes (IC) and TLR or IL-1R agonists, exert immunoregulatory functions and Th 2 responses. M2c macrophages, induced by IL-10 ou TGF-β, are associated to immunossupression, tissue remodeling and tumor promotion. Abbreviations: HLA-DR, human leukocyte antigen – DR isotype; MR, mannose receptor; TLR, Toll-like receptor. *Adapted from Mantovani et al., 2004* and *Martinez et al., 2014.*

3.5.6.3 Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are abundant in most human and experimental murine cancers [219]. Several epidemiological studies have showed a strong association between high TAM infiltration and advanced disease, worst prognosis and poor survival [220, 221], specifically in gastric cancer [222], breast cancer [223], melanoma [224], bladder cancer [225], pancreatic cancer [226], among others. In apparent contrast with the above results, high TAM infiltration has been associated with better prognosis in lung cancer [227] and CRC [228], despite of controversial reports [229, 230]. This differential impact of TAMs can be justified by the heterogeneity of macrophage populations inflammatory profile which should influence patient outcome.

TAMs are originated from circulatory monocytes (Ly6C^{High} macrophages) [231, 232], but also from spleen monocytes [114], which are continuously recruited to the tumors by a chemoattractant gradient created by tumor and stromal cells. Some of these recruiting

factors include the chemokines CCL2, CCL3, CCL4, CCL5, CCL8, CXCL12 as well as VEGF, placental growth factor (PGF), macrophage colony-stimulating factor (M-CSF), and angiopoietin-1 (reviewed in [233]). Specifically, CCL2 has been demonstrated to be crucial for monocyte recruitment in several tumors [234-236], and high CCL2 expression by tumors has been associated with poor prognosis [237, 238]. Accordingly, CCL2/CCR2 signaling inhibition stops the recruitment of inflammatory monocytes to tumor site, inhibiting metastasis formation and enhancing the survival of breast tumor-bearing mice [235]. Overexpression of M-CSF (CSF-1), the key regulator for macrophage differentiation, is associated with poor prognosis in a variety of cancers [239-241]. Studies using the polyoma middle T (PyMT) oncoprotein-driven mouse model of breast cancer showed that genetic ablation of CSF-1 reduces macrophage infiltration and inhibits metastasis formation [242]. Recently, Ries and colleagues reported that a monoclonal antibody for CSF-1 receptor (CSF-1R) reduces $F4/80^{+}$ TAMs and increases the $CD8^{+}/CD4^{+}$ T cell ratio in mouse animals. The administration of this inhibitor in diffuse-type giant cell tumor patients led to prominent decrease of the anti-inflammatory $CSF\text{-}1R^{+}CD163^{+}$ macrophages in tumor tissues, which translated into improved clinical objective responses [243]. These studies reinforced CCL2/CCR2 and CSF-1/CSF1R axis as potential targets for therapeutic intervention [244, 245].

Recent studies on the ontogeny of TAMs showed that they are, however, an heterogenous population, comprising blood, splenic but also tissue-resident macrophages derived from embryonic progenitors in brain, liver or lung [246]. For example, mouse lung tissue-resident interstitial macrophages contribute to the pool of TAMs, together with CCR2-dependent recruited macrophages, and impact on tumor progression in a different way. While resident TAMs correlated with tumor cell growth *in vivo,* the recruited TAMs accumulation was associated with enhanced tumor dissemination [247]. In a murine pancreatic ductal adenocarcinoma (PDAC) model, it was also reported a heterogenous TAM infiltration, being mainly constituted by tissue-resident macrophages derived from embryonic progenitors. Moreover, these cells expanded through *in situ* proliferation during tumor progression and exhibited a pro-fibrotic transcriptional phenotype [248]. In opposite, using a PyMT breast tumor model, TAMs but not mammary tissue macrophages (MTM) accumulated with increased tumor burden. TAMs depletion, but not MTM, their normal counterparts, restored tumor-infiltrating CTL response and suppressed tumor growth [232]. These studies strengthen the heterogeneity of macrophage populations within tissues and tumors, reflecting phenotypic and functionally distinct macrophages, creating new opportunities to cancer immunotherapy.

Upon recruitment of monocytes to the tumor, their polarization is susceptible to the variety of chemokines, cytokines and growth factors present at the tumor microenvironment. As they change over time, TAMs polarization and their functions depend on the tumor progression stage [249]. In early stages or regression stages of tumors, TAMs share many characteristics of M1-like profile, being associated with antitumor immunity activation and angiogenesis inhibition [250, 251]. In contrast, in advanced and relapsed tumors, TAMs are phenotypic and functionally closer to M2-like profile, dampening the immune activation [252, 253]. Probably, this shift is an attempt of cancer cells to evade the control of the immune system, by secreting anti-inflammatory cytokines, namely IL-10 and TGF-β, and VEGF, which will subvert macrophage polarization towards an immunosuppressive phenotype (Fig. 4). In fact, most of the studies associate TAMs infiltration with an M2-like profile [254], with anti-inflammatory, pro-angiogenic, and protumorigenic abilities [255]. These cells secrete low levels of IL-12 [256], required to activate an antitumor response mediated by NK cells, Th1 cells and CTLs; and low levels of iNOS [257], required for cytotoxic activities. Instead, TAMs produce high amounts of IL-10 and TGF-β, promoting Th2 and Treg cells differentiation/activation, dampening the antitumor immunity [258, 259]. More recently, TAMs were described to produce IL-6, IL-17, IL-23 cytokines which can promote cancer initiation and progression via STAT3 signaling in tumor cells [260, 261]. TAMs-derived PGE2 and IDO play important roles in the induction of immunosuppressive cells [262, 263], and their ability to secrete CCL17, CCL18 and CCL22 recruit Treg cells to the tumor site [264, 265]. Generally, TAMs express at their surface high levels of scavenger receptors (CD163 and CD204), mannose receptor (CD206) that have been explored for targeted therapies [266].

Currently, the essential role of macrophages in different stages of the tumor development, ranging from tumor growth, angiogenesis, invasion and metastasis is well established [267]. In different studies, TAMs infiltration positively correlates with tumor cell growth through the secretion of a variety of soluble factors, as IGF [268], HB-EGF [269], PDGF [270], IL-1, IL-6 [271, 272], and NADPH oxidase [273], which stimulate tumor cell proliferation and survival. TAMs have also an important role in the regulation of tumor angiogenesis. They are recruited to hypoxic areas and produce high amounts of pro-angiogenic factors, in response to a hypoxic environment, namely VEGF, PDGF, FGF, CXCL18, urokinase-type plasminogen activator, oncostatin M and eotaxin (Fig. 4) [274-279]. As a result, a tumor vasculature is formed, where tumor blood vessels are quite different from the normal vasculature, having an abnormal organization and morphology, irregular branching and chaotic distribution [280]. Their function as barrier is also compromised due to the abnormal distribution of endothelial cells and pericytes, which results in tumor vessel leakiness [281].

The relevance of TAMs in the angiogenic process was elaborated by Lin and colleagues who used the PyMT breast cancer model to demonstrate that TAMs are recruited to the tumor just before the angiogenic switch, and that macrophage infiltration inhibition delays vessel formation, as well as malignant progression [282]. Interestingly, De Palma *et al.* showed a subset of monocytes expressing the angiopoietin receptor Tie-2, named TIE-expressing monocytes (TEMs), that are recruited to the tumors and have potent pro-angiogenic properties and are essential for the angiogenic switch [283]. These macrophages establish interactions with activated endothelial cells along blood vessels, through angiopoietin-2 (ANG2), a Tie-2 ligand that works as chemoattractant for TEMs [284]. Targeting ANG2/Tie-2 axis, specifically by inhibiting ANG2 in endothelial cells, impairs TEMs-vessel association and inhibited angiogenesis and tumor progression. Additionally, Tie2 gene ablation in TEMs was enough to decrease tumor angiogenesis [285]. Tie2 deletion in myeloid cells also impaired blood vessels formation and tumor relapse after chemotherapy [286]. Recently, the presence of intratumorally TEMs was associated with a decreased overall and recurrence-free survival in PDAC and gastric patients [287, 288]. Overall, these studies instigate the scientific interest in anti-angiogenic therapies for cancer treatment [289].

TAMs are also described to have a key role in tumor cell abilities to migrate and invade the basement membrane, which ultimately reach through blood or lymphatic vessels distant sites, where they settle down and grow, creating metastasis [255]. TAMs through the production of high amounts of proteolytic enzymes, as MMPs and cathepsins [290-292] promotes the disruption of extracellular matrix and tumor cell invasiveness (Fig. 4). Multi-photon microscopy of mouse mammary tumors demonstrated that tumor cell intravasation occurs in association with TAMs [293]. Mechanistically, it was demonstrated that macrophages and breast tumor cells establish a paracrine loop, where tumor cells secrete CSF-1 to promote macrophage migration through the CSFR1 binding, and macrophages secrete EGF stimulating tumor cell migration and invasion by EGFR binding, in *in vitro* [294] and *in vivo* settings [295]. Additionally, inhibition of either CSF-1 or EGF-signaling reduces the migration of both cell types [295]. Furthermore, Yamaguchi and colleagues showed that EGF and CSF1 induce formation of ECM-degrading membrane protrusions, named invadopodia in cancer cells and podosomes in TAMs, facilitating their intravasation [296]. Our group provided new insights to this complex process by demonstrating that macrophages stimulate cancer cell invasion, motility and migration through enhanced MMPs activity and through EGFR activation. The ability of macrophages to promote tumor cell motility and invasion was impaired after EGF depletion [297]. In breast human cancers, TAMs-secreting CCL18 promotes cancer cells invasiveness by triggering integrin clustering

and their adherence to the extracellular matrix [298]. Accordingly, Pinto and colleagues demonstrated that decellularized human colorectal cancer matrices, derived from cancer patients' surgical resections, drive macrophages polarization towards an anti-inflammatory profile, with the ability to promote cancer cell invasion via CCL18 [299].

Interestingly, TAMs were also associated with EMT and tumor cell intravasation to circulation [300, 301]. Once tumor cells reach circulation, CTCs, may face difficulties to survive until reaching a secondary tissue, where they can install, growth and form secondary tumors or metastasis. In recent years, evidences that soluble factors, derived from primary tumor, prime specific tissues for tumor cell extravasations instigate the concept of the existence of a premetastic niche, a distant site from the primary tumor that has a favorable environment for tumor cell adhesion and invasion [129]. TAMs were also implicated in the formation of these premetastatic niches. Kaplan *et al.* showed that bone marrow-derived hematopoietic progenitor cells that express vascular endothelial growth factor receptor 1 (VEGFR1) form clusters in specific premetastatic sites before the arrival of tumor cells. Additionally, conditioned media obtained from distinct tumor types had a specific pattern of metastatic spread [302]. In accordance, tumor and stromal-derived premetastatic factors, including TNF-α, MMP9, TGF-β, CCL2, among others recruits bone marrow-derived cells to specific sites and promotes metastasis formation (revised in [303]). More recently, Wang *et al.* demonstrated that CRC cells secrete VEGF-A, which induces TAMs-producing CXCL1 at the primary tumor. Elevated CXCL1 levels in pre-metastatic liver recruited $CXCR2^{+}$-MDSCs to orchestrate the premetastatic site, which supported liver metastases [304]. Interestingly, macrophage binding to vascular cell adhesion molecule-1 (VCAM-1), overexpressed on breast tumor cells, contribute to tumor cells survival and lung metastases [305].

More recent studies have also demonstrated that macrophages can be associated with resistance to therapy and tumor relapse. Our group has recently shown that a cumulative dose of radiation of 10Gy (5*2Gy) induced a macrophage pro-inflammatory-like profile, but their pro-invasive and pro-angiogenic abilities were still preserved [306]. Studies with mammary cell lines co-cultured with macrophages suggested that macrophages-expressing cathepsin mediate resistance to chemotherapeutic paclitaxel, etoposide and doxorubicin. The combination of paclitaxel with cathepsin inhibition *in vivo* enhanced efficacy against breast primary and metastatic tumors [307]. Furthermore, preclinical studies demonstrated that perivascular M2 macrophages stimulate tumor relapse after chemotherapy [252].

All these studies regarding the role of macrophages on the tumorigenic process prompted the interest of macrophages as attractive targets for anticancer therapy, either alone or in combination with conventional treatments. Several efforts have been done to modulate the macrophages impact on the tumor microenvironment, either by inhibiting their recruitment, or by interfering with their survival, or inhibiting their signaling pathways, or by reprogramming them to a tumoricidal M1-like profile [308, 309]. These strategies have demonstrated promising results in preclinical models, and several therapeutic agents are currently under clinical investigation.

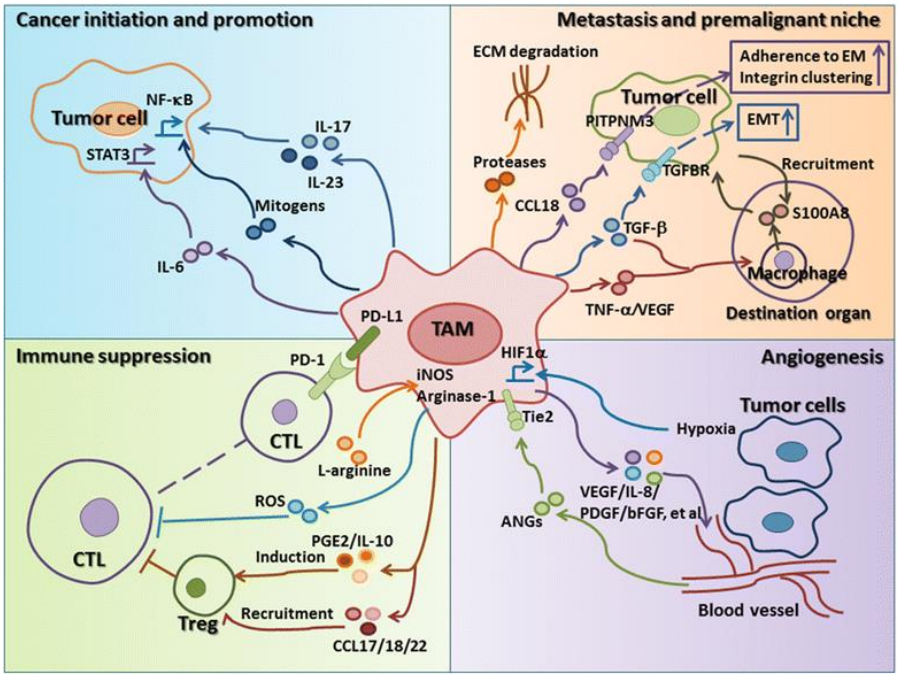

Figure 4. Tumor-associated macrophages role in tumor progression. TAMs are involved in several steps of tumorigenesis process, mainly through the secretion of several soluble factors, as cytokines, chemokines and MMPs. TAMs releasing factors as IL-6 and EGF contribute to tumor cell growth (blue panel). The secretion of IL-10, CCL17/18/22 promote Treg cells recruitment and function which further inhibits T cell functions. Moreover, TAMs expressing PD-L1 can directly inhibit T cell function (green panel). In response to a hypoxic tumor microenvironment, TAMs secrete several pro-angiogenic factors which acts on endothelial cells promoting abnormal and fenestrated tumor vascularization (purple panel). Lastly, TAMs contribute to metastatic process through ECM remodeling, epithelial-to-mesenchymal transition, and cell migration. *From Yang L. et al., J Hematol Oncol, 2017.*

4. Oncology meets Immunology

The tumor development is settled in an environment of chronic inflammation, characterized by an initial state of hypoxia, followed by interstitial and cellular edema, immune cells infiltration, formation of new blood vessels and tissue repair [310]. This inflammatory condition appears as a critical aspect of cancer progression, since it potentiates multiple

cancer hallmarks, including cell proliferation, cell death, angiogenesis, invasion and metastasis [5, 311]. However, the tumor-associated inflammation also represents an attempt of the host immune system to recognize and eliminate emergent cancer cells. This paradoxical role in promoting tissue homeostasis or tumor development has motivated several studies to understand which are the pro- and antitumor players, and how this balance can be managed to be favorable for the patients. But how is tumor-associated inflammation settled? Malignant lesions comprise aberrant proliferating cells with genomic damage that typically activate proliferation and survival signaling pathways, driving the expression of an inflammatory-related program orchestrated by specific transcription factors, as nuclear factor-kB (NF-kB) [312], STAT3 and hypoxia-inducible factor-1α (HIF-1α). Once these are upregulated by tumor cells, chemokines, cytokines, anti-apoptotic and cell survival factors, and proteolytic enzymes are secreted, leading to chronic engagement of innate immune cells (macrophages, DCs, neutrophils and NK cells), but also of stromal fibroblasts, vascular and mesenchymal cells. Innate immune cells recruitment contributes for the maintenance of the inflammatory microenvironment by producing high amounts of pro-inflammatory cytokines, namely TNF-α and IL-6, chemokines, MMPs and growth factors that further sustain stromal cells recruitment. Furthermore, these cells can secrete reactive oxygen species which also contribute for DNA damage, favoring a mutagenic environment. For example, excessive myeloid-derived hydrogen peroxide induces epithelial mutagenesis by triggering genome-wide DNA mutations [313]. These acquired mutations may result in immunogenic peptides which will be recognized in the context of major histocompatibility complex (MHC) class Ia on the surface of tumor cells by cytotoxic T cells, as non-self. APCs, mainly DCs and macrophages, orchestrates tumor antigens processing from dying tumor cells and present them at through MHC class II. These cells migrate through the lymphatic system to lymphoid organs and transport the information to naïve T cells, priming them and eliciting an effective T cell and B cell response.

4.1. Cancer Immunoediting

The concept that the immune system is fundamental for cancer destruction is not new. The tumor immune surveillance concept was firstly proposed by Lewis Thomas and Frank Burnet almost 60 years ago [314]. Later, it was demonstrated that the immune system has not only host-protecting but also tumor-sculpting actions, by promoting the outgrowth of less immunogenic tumor cell variants [315-317]. This dual effect of the immune system on tumor development instigated Robert and colleagues to redefine the cancer immunosurveillance hypothesis into one named cancer immunoediting (Fig. 5). They proposed that the cancer immunoediting process encompasses three phases: elimination, equilibrium and escape

[318]. In the Elimination phase, immune cells from the innate and adaptive arms of the immune system act together to eliminate tumor cells that escaped to damage repair mechanisms. Nevertheless, there is a possibility that a portion of these tumor cells can survive and be kept in a dormant state, coexisting with immune cells in a delicate equilibrium. The molecular mechanisms involved in the immune-mediated tumor dormancy are not well understood. Still, a balance between antitumor (IL-12, IFN-γ) and protumor (IL-10, IL-23) cytokines maintains tumors in Equilibrium [319]. At this stage, some tumor cells undergo genetic and epigenetic modifications to overcome the constant selection pressure caused by the presence of immune cells. Several immune strategies acquired by resistant tumor cells have been associated with their ability to evade immune destruction (Fig. 5). These include: i) the loss of tumor antigens, MHC class I or costimulatory molecules, ii) the expression of transcription factors involved in tumorigenesis and resistance (STAT3), iii) expression of anti-apoptotic (bcl2) and immunosuppressive molecules (IDO, PD-L1, galectin-1,3,9), iv) secretion of VEGF, TGF-β, IL-6, M-CSF, which promote angiogenesis and maintenance of an immunosuppressive microenvironment. Furthermore, the myeloid cells recruited to the tumor site, namely, TAMs, DCs and MDSCs, may also express immunoregulatory molecules, as ARG1 and IDO, and secrete anti-inflammatory cytokines, IL-10 and TGF-β, which induce the differentiation and activation of Treg cells, inhibiting T cell-mediated cytotoxicity. T cells can also express inhibitory receptors such as PD-1, CTLA-4, Tim-3 and LAG-3 that inhibit antitumor immunity and promote tumor growth [320]. Thus, during the Escape phase, the balance is skewed towards tumor progression and the immune system is no longer able to contain the progressive tumor growth, with resistant tumor cells invading adjacent tissues and establishing metastasis.

Clinical observations also evidenced the role of the immune system in the control or in the promotion of tumor development. Studies showed that patients with primary immunodeficiency had higher risk to develop lymphoproliferative disorders, as non-Hodgkin´s lymphomas, Hodgkin´s disease and leukemia [321]. Patients that received transplants had three times the number of neoplasms when compared with age-matched controls, due to immunosuppressive treatments [322]. Patients with tumors infiltrated with high number of lymphocytes, namely T cells ($CD3^+$), CTLs or memory T cells had, in general, better prognosis. Conversely, immune suppressive populations as MDSCs, macrophages and Th2 and Treg cells are frequently associated to poor prognosis, accordingly the tumor type [323].

Considering the great advances in the immune-oncology field, namely in the understanding of the ability of the immune system to recognize and destroy tumor cells, combined with the

increasing knowledge about strategies for tumor cells evade the immune system, a new era in anticancer therapy emerged.

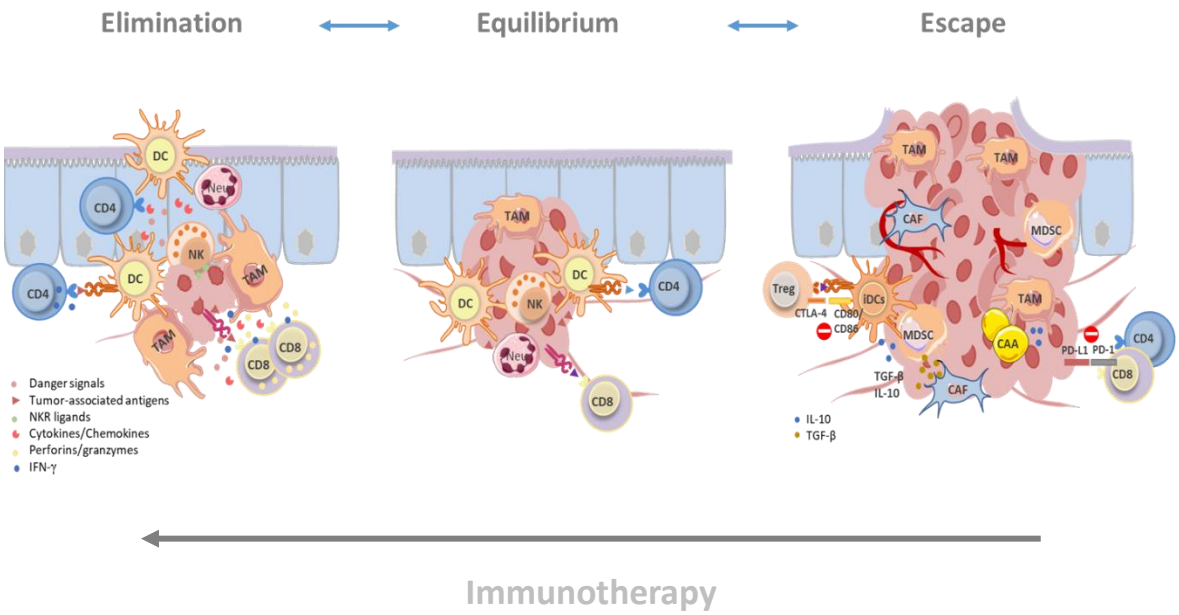

Figure 5. Cancer immunoediting: from immunosurveillance to immune escape. The cancer immunoediting includes three stages: the elimination, the equilibrium and the escape. The elimination phase usually occurs in early stages of tumorigenesis, where highly antigenic clones are recognized by innate immune cells which activate the adaptive system, leading to tumor cell killing. In the equilibrium phase, tumor and immune cells coexist, being the tumor outgrowth controlled. Tumor cell variants which have acquired resistance to elimination then enter the escape phase, namely by decreasing MHC I and by releasing immunosuppressive cytokines. These resistant variants will escape the immune system control, proliferate, and eventually metastasize. *Adapted from Kalbasi A. et al., JCI, 2013.*

4.2. Cancer Immunotherapy

The development of novel immunotherapeutic strategies revolutionized cancer treatments. Most of them aim to potentiate CTL activities, as immune checkpoint inhibitors, adoptive T cell therapy and cancer vaccination. Nevertheless, several strategies targeting immunosuppressive myeloid cells are currently in clinical trials. These strategies will be here briefly explored.

4.2.1. Counteracting T cell anergy/immunosuppression

4.2.1.1. Adoptive T cell therapy

Adoptive T cell transfer therapy used either *ex vivo*-expanded autologous reactive tumor-infiltrating lymphocytes (TILs) or genetically engineered ones, to express high affinity T cell receptors or chimeric antigen receptors, have shown encouraging results in several types

of cancer. Specifically, adoptive transfer of TILs has shown to induce long-lasting responses and to complete regression of metastatic melanoma. The combination of adoptive transfer of autologous TILs administered with IL-2, following a lymphodepleting preparative regimen, resulted in 56% of clinical responses, and 22% of melanoma patients experienced complete tumor regression [324]. The regression achieved with adoptive TILs transfer was associated with a higher number of infiltrated tumor-reactive T cells [325]. These optimistic results instigated the interest in autologous TILs transfer for combination therapy, as anti-CTLA-4, anti-PD-1, BRAF inhibitor [326-328] and for other solid tumors [329, 330]. Nevertheless, melanoma appeared to be the only cancer where autologous TILs efficiently recognize tumor antigens, inducing significant tumor regression [331]. To overcome this limitation several strategies engineering lymphocytes have been developed.

Autologous peripheral blood lymphocytes (PBL) that were gene transduced to express antigen-specific cell receptors represents a promising therapy to provide widely tumor-specific immunity to cancer patients [332-334]. Normally, this antigen-specific recognition is mediated by αβ T cell receptors but currently the main encouraging therapeutic strategy is based on chimeric antigen receptor (CAR) development. This strategy consists in the construction of an antibody single chain variable fragments joined with TCR and co-stimulatory molecules that will recognize the specific antigen in an MHC-independent context. In fact, two autologous CAR T cell therapies, namely Kymriah™ and Yescarta™ were approved last year. They are both CD19-specific CAR T cell therapies and are permitted by Food and Drug Administration (FDA) for treatment of refractory or relapse B cell precursor acute lymphoblastic leukemia and refractory or relapse B cell lymphoma, respectively [335]. CAR T cell therapy has not been effective in solid tumors [336, 337] likely due to the locally immunosuppressive environment, observed in many solid tumors. Therefore, the modulation of the immunosuppressive tumor microenvironment with immune checkpoint inhibitors might boost the efficacy of adoptive T cell therapy in general [338, 339]. Despite the many promising beneficial results taking in consideration that these therapies are applied in patients resistant to many other therapies, adoptive T cell therapy also has its limitations. As a personalized immunotherapy, T cell infusion have to be produced for every individual patient, with costs relatively high, and the success rates of T cells outgrowth is variable. Furthermore, given the required manipulations, the production of a T cell infusion is longer than one month, which may be too late for patients with progressive disease [328].

4.2.1.2. Immune Checkpoint Inhibitors

The blockade of immune checkpoint molecules has appeared as an attractive strategy to potentiate antitumor immunity and treat cancer patients. Once T cells are activated, they upregulate on their surface inhibitory receptors, as CTLA-4 and PD-1, which frequently maintain self-tolerance and limit tissue damage during pro-inflammatory responses. However, their expression can be engaged by cancer cells as a mechanism to evade immune destruction [340]. CTLA-4 is an intracellular protein in resting T cells. Upon TCR and CD28 engagement, CTLA-4 translocates to the surface and competes with CD28 to bind costimulatory molecules, such as CD80 and CD86, resulting in arrest of T cell proliferation and activation [341-343]. CTLA-4 is mainly expressed on $CD4^+$ T cells and, therefore, the increased CD8 T cell responses in patients treated with anti-CTLA-4 is likely induced by $CD4^+$ T cell activation [344]. Furthermore, CTLA-4 plays an important role on Treg cells immunosuppressive functions [345]. As CTLA-4 stops potentially T cells at the initial stage of naïve T cell activation, the immunosuppressive activities of CTLA-4 blockade seem reside mainly in secondary lymphoid organs.

Similarly, PD-1 is absent on naïve T cells and is expressed upon TCR binding. The two ligands for PD-1, PD-L1 and PD-L2, are differently regulated [346, 347]. While PD-L1 is induced on activated hematopoietic cells and on epithelial cells by IFN-γ-secreted by activated T cells and NK cells, as a mechanism to limit the immune response, PD-L2 is highly selective expressed on activated DCs and on macrophages, induced mainly by the presence of IL-4 than of IFN-γ [322, 323]. PD-1 binding results in PD-1–mediated T cell exhaustion, inhibiting the antitumor cytotoxic T cell response [348]. In opposite to CTLA-4, PD-1 blockade seems to work predominantly at the tumor microenvironment, where its ligands are overexpressed by APCs and tumor cells [349], suggesting that PD-1 and CTLA-4 play complementary roles in the regulation of the adaptive immunity [350]. These immune inhibitory properties encouraged the design of monoclonal antibodies for CTLA-4 (Ipilimumab), PD-1 (Nivolumab, Pembrolizumab, Cemiplimab) and PD-L1 (Avelumab, Durvalumab, Atezolizumab), which have successfully generated antitumor immune responses in some cancer patients [351]. Clinical activity of CTLA-4 blockade was most clear in patients with advanced metastatic melanoma, with 15% of objective response and 16% of patients with durable response for more than 10 years [352, 353]. Given the high rate of inflammatory side effects in response to anti-CTLA-4 therapy and relatively low response rate, the identification of predictive biomarkers that may discriminate responders from non-responders patients is a priority. As example, patients with genomic defects in the IFN-γ pathway are identified as non-responders to ipilimumab [354]. Additionally, analysis of melanoma samples from responders and non-responders patients to anti-CTLA-4

therapy evidenced that a higher tumor mutational burden is associated with clinical benefit [355]. Regarding anti-PD-1/PD-L1 axis, six inhibitors are currently approved by FDA to treat 11 types of cancer [356]. PD-1 blockade, or anti-PD1-based combination is currently the first-line therapy in advanced melanoma patients, with 50% of the patients showing an unprecedent 3-years overall survival [357]. Considering the distinct immunological mechanisms mediated by CTLA-4 and PD-1, some clinical trials combined nivolumab and ipilimumab and the response to the therapy was better than comparing with single treatments in advanced melanoma patients [358]. Additionally, most of the patients treated with anti–PD-1 or anti–PD-L1 antibodies have no severe side effects, and treatment-related deaths are very rare.

4.2.1.3. Cancer Vaccines

Cancer therapeutic vaccines, unlike prophylactic vaccines that are designed to prevent disease in healthy individuals, are administrated to cancer patients to eliminate cancer cells through eliciting immune responses [359]. They are a type of immunotherapy formulated with tumor antigens, which can be tumor-associated or tumor-specific (neoantigens), and with immune adjuvants, such as poly:IC, monophosphoryl lipid A, CpG, imiquimod, among others. The formulation can be variable, as protein or peptide-based, nucleic acids-based, cell-based, as antigen-loaded autologous DCs; and vector-based [360]. The delivery vehicles, currently in clinical trials, include liposomes, emulsion, virosomes and nanodiscs. The first cancer vaccine FDA approved, already in 2010, was an autologous DC vaccine, (Sipuleucel-T™) destined for metastatic castration-resistant prostate cancer (mCRPC), with the ability to prolong patient survival in 4.1 months, in comparison to placebo [361]. PROSTVAC™ is a genetic cancer vaccine also for mCRPC, which consists in poxvirus-based vectors to provoke effective immune responses against the prostate specific antigen and improved in 8.5 month the median overall survival [362]. Oncovax™ cancer vaccine contains irradiated, non-tumorigenic autologous tumor cells with Bacillus Calmette-Guérin and had success in early phase clinical trials with improvement in disease-free and overall survival in CRC patients [363]. Despite the preclinical studies have provided broad evidences of the benefit of cancer vaccines, clinical translation has proved to be challenging. The recent advances in immune checkpoint inhibitors can be valuable for combinatory therapies with cancer vaccines.

Overall, it is clear the benefits of immunotherapy for cancer patients, but unfortunately only a small percentage of them benefit with a long-term tumor remission. This is partially caused by the presence of immature and/or immunosuppressive myeloid cells at the tumor microenvironment, which support the ability of tumor cells to subvert the immune recognition

and escape to tumor destruction. Additionally, the absence of biomarkers to discriminate the therapy responders from non-responders patients remains a challenge as well as the high costs associated with long periods of therapy. Therefore, all these questions continue to be the main drawbacks for the actual immunotherapeutic strategies and reinforce the importance of the combination of different therapies.

4.2.2. Counteracting immunosuppressive/tolerogenic myeloid cells

Myeloid cells are a major immune component present in the most solid tumors. In more advanced cancers, the tumor microenvironment recruits e reprograms the profile of macrophages, DCs, MDSCs and neutrophils towards an immunosuppressive profile to sustain tumor progression. Specifically, macrophages and MDSCs, seem to be the main players of these immunosuppressive activities, being frequently associated with poor prognosis [221, 364]. Furthermore, these cells have been associated with resistance to conventional therapies [365, 366]. For example, the resistance associated to PI3K inhibitors in solid tumors is associated with an increased infiltration of macrophages in preclinical models [367]. TAM infiltration decreased drug-induced apoptosis through IL-6/STAT3 activation and was associated with chemoresistance in CRC patients [368]. Additionally, the resistance of CRC patients to the chemotherapeutic drug 5- fluorouracil (FU) was related with TAMs-secreting putrescine, a polyamine, inducing resistance to 5-FU-triggered CRC apoptosis [369]. Regarding MDSCs, it was recently reported that MDSCs-related microRNAs are an indicator of MDSC activity and predict immunotherapy resistance in melanoma [370]. Therefore, therapeutic strategies targeting myeloid cells appear as very promising, since they can potentiate cancer cell toxicity while activating specific T cell-mediated immunity. TAM-targeted therapies including their depletion, inhibition of their effector functions, or functional reprogramming to an antitumor phenotype, have been explored and different degrees of efficacy were noticed, as further discussed.

4.2.2.1. TAM-targeted therapies

Most macrophage-based therapies are currently focused on CSFR1 inhibitors, which either deplete or reprogram macrophages, depending on the cancer context (Fig. 6). For example, in breast tumors, CSF1R blockade reduced macrophage infiltration, improving CTL response and response to chemotherapy [371, 372]. Instead, in brain tumors, CSF1R blockade re-educated macrophages towards an antitumor profile, blocking tumor progression [373]. More recently, Quail and colleagues showed, in a glioma preclinical model, resistance to CSF1R blockade, mediated by PI3K hyperactivation. This phenotype was driven by macrophage-derived IGF1 and IGF1 receptor (IGF1R) upregulated on tumor

cells, resulting on subsequent PI3K activation. Notably, combining PI3K or IGF1R inhibitors with CSF1R blockade prolonged tumor-bearing mice overall survival [374]. Recent results from clinical studies in glioblastoma multiforme, using CSF1R inhibitors evidenced limited efficacy to date, with no alterations on patient's progression free-survival over a 6-month of follow-up [375]

Another rationale of targeting macrophages is to reprogram their immunosuppressive and protumoral phenotype at the tumor microenvironment, enhancing T cell-mediated antitumor activities. Beatty and colleagues showed in a mouse model of PDAC that CD40 activation, a molecule involved in immune activation, could disrupt the immunosuppressive microenvironment, increasing macrophage MHC class II and CD86 expression and their infiltration into the tumors, rendering an antimoral phenotype [376]. Patients with incurable PDAC treated with the combination of an agonist CD40 antibody with gemcitabine evidenced therapeutic benefit [377]. More recently, it was demonstrated that a single dose of CD40 antibody with chemotherapy rendered PDAC mouse model susceptible to T cells-mediated killing and potentiated durable remissions [378]. Consistently, Zhu and colleagues, using a mouse model of PDAC showed that CSF1R signaling blockade reprogram macrophage responses, enhancing antigen presentation, antitumor T cell responses, but still upregulating T-cell checkpoint molecules. The combination treatment with CSF1R antibody and immune checkpoint inhibitors enhanced efficacy compared to checkpoint inhibitor treatment alone [379]. Importantly, in a mouse model of $BRAFV^{600E}$-driven melanoma, CSF1R blockade improved the efficacy of adoptive T cell therapy by inhibiting the accumulation of TAMs and skewing their profile towards an immunostimulatory one [380]. These results highlight the importance of evaluation of tumor macrophagic populations abundance and phenotypes to better predict which patients will better respond to T cell-based immunotherapies.

Another strategy targeting macrophages is to prevent their recruitment to inflamed tissues through blockade of chemokine gradients (Fig. 6). One classical example is the blockade of the CCL2/CCR2 axis. Qian and colleagues showed that $Gr1^{+}$-macrophages-expressing CCR2 infiltrate lung metastasis in mouse and human breast tumors, and that inhibition of CCR2/CCL2 signaling blocked the recruitment of these inflammatory macrophages to the lungs, inhibiting metastasis formation *in vivo* and prolonging the overall survival of tumor-bearing mice [235]. Nevertheless, others reported that the interruption of CCL2 inhibition boosted metastasis formation, mediated by an increase of monocytes and cancer cells mobilization from the primary tumor, as well as increased blood vessel and metastasis formation in an IL-6 and VEGF-α-dependent manner [381]. Other possible strategy is through the inhibition of the CXCL12/CXCR4 axis. Boimel and colleagues have shown that

cancer cell secreted CXCL12 increased macrophage infiltration, microvessel density and *in vivo* invasion [382]. Recently, it was described that CCR4 antagonist have no impact in the proportion of infiltrating leukocytes but altered the phenotype of myeloid cells, increased NK cell and Th1 cytokine levels [383]. Nevertheless, caution should be taken since high levels of CXCL12 are known to maintain HSCs and progenitor cells within bone marrow reservoirs [384].

Strategies targeting Tie2-macrophages which are involved in angiogenesis process appears also as viable way to disrupt tumor progression (Fig. 6). In fact, Tie2-macrophages are required for blood vessel rebuilding and tumor relapse after chemotherapy [286]. Tie2-macrophages depletion in different cancer models is associated with decreased angiogenesis and delayed tumor growth [385, 386]. Nevertheless, in the light of previous described compensatory mechanisms to VEGF blockade, we can anticipate that hypoxia resulted from Tie2-depletion can boost pro-angiogenic factors and vascular remodeling.

Lastly, IFNs and TNF-α are also being reported as able to reprogram macrophages towards an immunostimulatory profile and are currently tested in clinical trials in combination with radio-, chemo- and immunotherapies (Fig.6) [387, 388]. As the IFN-γ therapy is under focus on this thesis, the role of this cytokine on tumor development and therapy will be reviewed in the next chapter. Overall, the majority of the clinical trials ongoing are in phase I and phase II, testing the tolerability and the efficacy of the treatments in cancer patients. Perhaps the strategies aiming reprograming macrophages appears from preclinical models as the most appealing and safety ones.

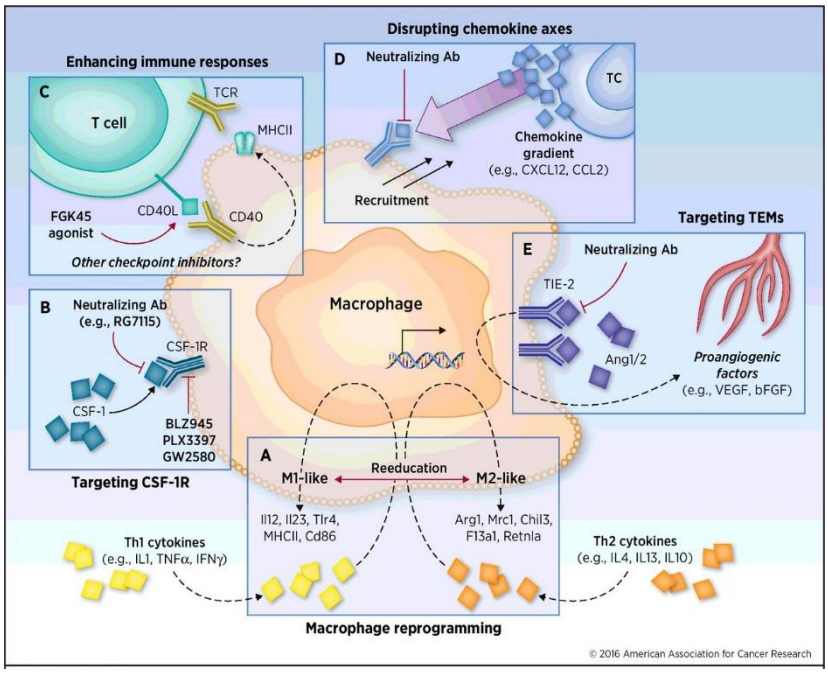

Figure 6. Strategies targeting macrophages in cancer. Several approaches have been considered to target macrophages in tumor microenvironment, specifically (A) reprograming macrophage polarization from M2-like profile towards M1-like; (B) neutralization of CSF-1R which deplete or reprogram macrophages depending on the tumor context; (C) enhancing the macrophage antigen presentation ability to promote antitumor T cell response; (D) prevent macrophage recruitment through blockade of chemokine gradients, namely SDF-1/CXCR4 and CCL2/CCR2; (E) blocking specific macrophage activities, namely the inhibition of pathways involved in their pro-angiogenic role, to reduce tumor vascularization. *Reprinted from Quail DF et al, Clin Canc Res, 2017.*

5. New therapies to harness the immune system

Recent advances in cancer immunotherapy highlighted the relevance of immune system in cancer control and elimination. Despite have serious benefits in a small percentage of patients, the low immunogenicity and the off-target side effects remain challenges for a widely effective cancer immunotherapy. Cancer nanomedicine has been emerging in recent years, firstly by offering alternatives for chemotherapeutic drugs delivery and, more recently, for appealing features for diagnosis and imaging, synthetic vaccine development and, as well as the immunomodulatory nature of some biomaterials. Several advantages associated with the use of these nanostrategies have been reported, such as: i) the improvement of drug therapeutic index either by increasing its efficacy and/or reducing side effects; ii) the enhancement of drug pharmacological properties (for example, stability, solubility, circulating half-life and tumor retention); iii) targeting delivery of drugs in a cell-specific manner; iv) sustained or stimulus-activated drug release, enabling biomacromolecular compounds delivery in targeted sites (for example, small interfering RNA (siRNA), DNA, mRNA, and proteins); v) co-delivery of multiple drugs to improve therapeutic efficacy and overcome drug resistance; vi) drug transcytosis across epithelial and endothelial barriers; vii) more accuracy in cancer diagnosis and imaging; viii) real-time feedback of nanotherapeutic agent efficacy *in vivo*; and ix) innate therapeutic properties of some biomaterials which can be combined with targeted and drug delivery [389].

Some cancer nanotherapies are already in clinical use, and others evidenced great potential in clinical trials [389]. The first nanoparticle (NPs)-based cancer treatment approved by FDA, in 1995, was Doxil, a formulation containing liposomes and the chemotherapeutic agent, doxorubicin. Its main benefit was the prolonged drug circulation time and reduced side effects compared to free doxorubicin [390]. Afterwards, several liposome-based nanotherapies for the delivery of chemotherapeutic agents were approved for several cancers, including metastatic breast cancer, ovarian cancer and multiple myeloma. Although these strategies have been improved the pharmacokinetics and biodistribution of the encapsulated drugs, the patient overall survival was not significantly altered, in contrast

with what was observed in animal models [391]. Importantly, most of the nanostrategies used in solid tumors treatment are systemically administrated and accumulate in the tumor through the enhanced permeability and retention (EPR) effect. The enhanced permeability of the tumor vessels allows the accumulation of drugs at the tumor site, enabling NPs entry into the tumor interstitial space, while the poor lymphatic drainage provoke retention within the tissue [392]. The EPR effect was the groundwork of nanostrategies delivery to tumors [393, 394]. Nevertheless, the interpretation of EPR effect has been oversimplified. While the EPR varies considerably between patients and tumor types, several factors can affect the accumulation of NPs at the tumor, such as i) the interaction with serum proteins, ii) biodistribution, iii) extravasation and interaction with the tumor microenvironment and, iv) tumor/stromal cell targeting and internalization. Additionally, the physicochemical properties of the NPs can also influence these biological interactions, impacting the EPR effect and the therapeutic outcome [395].

In addition to chemotherapeutic agents' delivery, cancer nanomedicine is currently centering its focus in the immunotherapeutic field, since the recent advances in the area showed that the modulation of immune system can potentiate an effective antitumor response. Thus, nanomedicine is being investigated to boost the effectiveness of immunotherapy [396]. The nanostrategies designed to potentiate the immune activation, namely for macrophages and DCs, will be briefly discussed below.

5.1. Nanomedicine targeting TAMs

Macrophages have been appointed as ideal targets of NPs since they are virtually in all tissues of the body and are specialized in the internalization of foreign material [397]. Furthermore, their phenotypic and functionality plasticity makes them attractive targets for reprogramming towards an antitumor profile. In fact, NPs have been recognized to localize with both primary and metastatic tumors following systemic administration [398, 399]. More exactly, Miller and colleagues showed that NPs have been found within TAMs and healthy macrophages upon systemic administration [400]. Some studies have demonstrated that TAMs uptake NPs acting as a local drug depot. Interestingly, TAMs depletion reduced intratumoral NPs accumulation and their therapeutic efficacy [401]. Recently, Miller and colleagues elaborated an elegant study where they evidenced that local tumor irradiation substantially increases TAMs relative to tumor cells and, thus, NPs delivery [402]. Overall, these studies driven the interest of NPs as therapeutic agents to reprogram macrophage profile at the tumor microenvironment.

Macrophage response to NPs is affected by several factors, including NPs size and composition, their surface characteristics and overall charge, route of administration and

therapeutic dose. Upon enter in the body, they are generally recognized and internalized by macrophages, and if these NPs are not inert, macrophages will react to their uptake by undergoing polarization [403, 404]. However, the exact contribution of specific NPs properties to the polarization profile is not completely understood. Based on NPs core composition, silica [405, 406], gold [407, 408], polymeric [409, 410], cationic polymers [411-413], carbon [414-416], metallic [417] and iron oxide-based NPs have been described to induce a M1-like profile while liposomal-based NPs [418, 419] were associated with M2-like profile [420]. Iron oxide NPs (Ferumoxytol) are currently FDA approved as an iron supplement and they have been under focus due to their stronger antitumor and immunostimulatory effects, when compared to other NPs. Once iron handling and macrophage polarization are intrinsically connected [421], several studies have reported the impact of iron oxide NPs on macrophage polarization. Zanganeh *et al.* demonstrated that macrophages treated in *in vitro* with ferumoxytol are associated with pro-inflammatory Th1 response. Furthermore, ferumoxytol-treated mice showed a reduced tumor growth of subcutaneous adenocarcinomas by increased accumulation of M1 macrophages in the tumor tissue. Liver metastasis formation was also prevented by intravenous ferumoxytol treatment previous intravenous tumor cell inoculation [422]. Similarly, albumin-bound paclitaxel NPs (Abraxane, FDA approved in 2005) have also been associated with M1-like polarization, with an increase in pro-inflammatory cytokine production comparing to paclitaxel alone. This effect was abrogated in the presence of TLR4 inhibitors [423]. Hyaluronic acid (HA) coated, mannan-conjugated MnO2 particle (Man-HA-MnO2) combined with doxorubicin inhibited tumor growth and tumor cell proliferation, as compared with chemotherapy alone, by priming TAMs towards to M1-like profile and attenuating tumor hypoxia [403]. Others also shown that Fe_3O_4-doxorubicin-HA NPs enhanced antitumor efficacy through the combinatorial action of Fe_3O_4 NPs, doxorubicin and HA, which promoted M1 polarization, tumor cell killing and tumor and TAM-targeting, respectively [424]. Interestingly, Rodell and colleagues showed that β-cyclodextrin NPs loaded with R848 (CDNP-R848), agonists of TLR7 and TLR8, was a potent driver of M1 polarization *in vitro*. Additionally, CDNP-R848 administration in multiple mice tumor models shift macrophage profile towards a pro-inflammatory M1-like, leading to tumor growth control and protecting animals against tumor rechallenge. When CDNP-R848 were combined with anti-PD-1 therapy, the response rates were even better than in single treatments [425]. Overall, these studies showed the potential of rationally engineered drug-nanoparticle combination to efficiently modulate TAMs for cancer therapy.

5.2. Nanomedicine targeting DCs

DC targeting constitutes also an appealing strategy to avoid the high cost and complexity associated with autologous DCs cancer vaccines. Additionally, the development of NPs allows to target specific DCs subsets, potentiating the stimulatory capacity of specific DCs populations. These NPs can also be conjugated with tumor-associated antigens (TAAs), RNA encoding TAAs, immune adjuvants, such as TLR ligands, cytokines, and siRNA, further improving DCs activation and consequently, effective T cell response. Importantly, co-delivering of TAAs and immune adjuvants within the same system will ensure that only DCs exposed to antigen receive the activation signal. Antigens carried by NPs can enter in DCs via receptor-mediated endocytosis or pinocytosis [426] and their immune response is impacted by NPs size, composition, surface charge and route of administration [427].

Several NPs formulations, such as liposomes, poly(lactic-co-glycolic acid) (PLGA), or poly-γ-glutamic acid (γ-PGA) NPs have been used to target DCs, and their impact on DCs profile and T cell stimulatory capacity have been extensively described. For example, γ-PGA NPs induced high expression of DCs maturation markers, eliciting Th1 cell responses in a TLR4 and MyD88-dependent manner [428], and this capability was dependent from the NPs size [429]. Ovalbumin or listeriolysin-carrying γ-PGA NPs induced antigen specific CTL activity and IFN-γ production [430]. These studies suggest γ-PGA NPs have not only an antigen-carrying capacity but also a potent adjuvant function of eliciting adaptive immune responses. Chitosan and PLGA films induce DCs maturation while alginate or hyaluronic acid films decreased the expression of costimulatory molecules [431, 432]. Importantly, NPs have multiple applications since they can be conjugated with specific antibodies or peptides to bind surface DCs receptors. For example, DEC-205 and DC-specific intercellular adhesion molecule 3-grabing nonintegrin (DC-SIGN) are often used as target molecules for nanostrategies targeting DCs [433, 434].

Other nanostrategies targeting CD11c and CD40 have been also explored. In fact, targeted-PLGA NPs combined with ovalbumin and TLR3 and 7 ligands (PLGA/OVA/TLR37L) were more efficiently internalized and had higher efficacy in stimulating CD8+ T cell responses, in comparison to the control non-targeted NPs [435]. Mannose-functionalized polymeric NPs combined with TLR3 and TLR9 ligands induced Th1 cell responses, decreasing melanoma tumor growth in therapeutic and prophylactic settings. Additionally, the combination of mannose-functionalized NPs with TLR ligands and MHC class I or II-restricted melanoma antigens induced highest tumor growth delay [436]. Combinatorial NPs targeting both DC-SIGN and $BDCA3^{+}$ DCs enhanced T cell activation, in comparison to targeting either DC subset alone, via IL-15-dependent [437]. Recently, Min and colleagues evidenced that several formulations of antigen-capturing NPs deliver tumor-specific proteins

to APCs, improving the efficacy of anti-PD-1 therapy in mice melanoma model, by increasing $CD8^+$ T cell response and increased both $CD4^+$T/Treg and $CD8^+$T/Treg ratios [438]. Although DCs-targeted NPs have not yet entered in clinical trials, their use as vaccine adjuvants is being under clinical investigation [439, 440].

Overall, these emergent nanotherapies showed that through myeloid cells reprogramming, an effective antitumor immune response can be achieved. Additionally, by priming the innate immunity, these nanostrategies may potentiate the efficacy of conventional therapies and immunotherapy. Similar approaches combining immunomodulators/nanostrategies and conventional therapies are currently under clinical investigation, and it is expectable that they will become a reality in the foreseeable future.

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CHAPTER II

Interferon-gamma at the crossroads of tumor immune surveillance or evasion

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REVIEW
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Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion

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Interferon-gamma (IFN-γ) is a pleiotropic molecule with associated antiproliferative, pro-apoptotic and antitumor mechanisms. This effector cytokine, often considered as a major effector of immunity, has been used in the treatment of several diseases, despite its adverse effects. Although broad evidence implicating IFN-γ in tumor immune surveillance, IFN-γ-based therapies undergoing clinical trials have been of limited success. In fact, recent reports suggested that it may also play a protumorigenic role, namely, through IFN-γ signaling insensitivity, downregulation of major histocompatibility complexes, and upregulation of indoleamine 2,3-dioxygenase and of checkpoint inhibitors, as programmed cell-death ligand 1. However, the IFN-γ-mediated responses are still positively associated with patient's survival in several cancers. Consequently, major research efforts are required to understand the immune contexture in which IFN-γ induces its intricate and highly regulated effects in the tumor microenvironment. This review discusses the current knowledge on the pro- and antitumorigenic effects of IFN-γ as part of the complex immune response to cancer, highlighting the relevance to identify IFN-γ responsive patients for the improvement of therapies that exploit associated signaling pathways.

Keywords: type II interferon, immunoregulation, cancer microenvironment, immunotherapy, immune contexture

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INTRODUCTION

Interferons (IFNs) are pleiotropic cytokines with antiviral, antitumor and immunomodulatory properties, being central coordinators of the immune response (1). The term "interferons" comes from the description of molecules protecting cells by "interfering" with viral infection (2, 3). Three major types of IFNs are distinguished by their sequence identity, genetic loci, cell of origin, nature, and distribution of their receptors and resulting stimuli (**Table 1**).

The human type I IFN family comprises 17 distinct proteins, mainly represented by IFN-α and IFN-β, which are ubiquitously expressed and signal through their cognate receptor, composed by IFNαR1 and IFNαR2 subunits [reviewed in Ref. (4)]. IFN-γ is the lone member of type II IFN family. It is more restrictively expressed and is structurally and functionally different from the other types of IFNs. Most recently, a type III IFN family was described to be composed of four homologous proteins (IFNλ1–4), which bind the IFNλR1 and interleukin (IL)-10Rβ heterodimeric receptor [reviewed in Ref. (8)]. To date, type I and type III IFNs have been mainly involved in host–pathogen

TABLE 1 | Comparison of human type I, type II, and type III IFN production and signaling.

Properties	Type I IFN (IFN-α, IFN-β)	Type II IFN (IFN-γ)	Type III IFN (IFN-λ)
Members	17 proteins: 13 IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω	1 protein: IFN-γ	4 proteins: IFN-λ1, IFN-λ2, IFN-λ3, IFN-λ4
IFN-producing cells	All nucleated cells	T cells, B cells, NK cells, NKT cells, and APCs	All nucleated cells, mainly mDCs, pDCs, and epithelial cells
IFN-responding cells	All nucleated cells	All nucleated cells	Lung, intestine, and liver epithelial cells
Stimuli	DAMPs and PAMPs	IL-12, IL-15, IL-18, type I IFN, and PAMPs	DAMPs and PAMPs
IFN receptor	IFN type I receptor (IFNαR): IFNαR1 and IFNαR2 subunits	IFN type II receptor (IFNγR): IFNγR1 and IFNγR2 subunits	IFN type III receptor (IFNλR): IFNλR1 and IL10Rβ
Signaling molecules	TYK2, JAK1, all STATs, CRKL, and IRS	JAK1, JAK2, STAT1, and STAT3	TYK2, JAK1, STAT1, STAT2, and IRF9
Transcription factor binding sites	ISRE (canonical) GAS (non-canonical)	GAS (canonical) ISRE (non-canonical)	ISRE
Functions	Antiviral, antiproliferative response, regulation of cell survival/apoptosis, and immunoregulation	Antiviral, antiproliferative, immunomodulatory, and antitumor response	Antiviral response, mucosal immunity
Reference	(4, 5)	(6, 7)	(8)

APCs, antigen-presenting cells; CRKL, CT10 regulator of kinase-like; DAMPs, damage-associated molecular patterns; GAS, gamma-activated site; IFN, interferon; IFN-γ, interferon-gamma; IFNαR1–2, type I receptor; IFNγR, type II receptor; IFNλR, type III receptor; IL, interleukin; IRF, interferon-regulatory factor; IRS, insulin receptor substrate; ISRE, interferon-sensitive response element; JAK, Janus kinase; mDCs, myeloid dendritic cells; NK, natural killer; NKT, natural killer T cells; PAMPs, pathogen-associated molecular patterns; pDCs, plasmacytoid dendritic cells; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase.

interactions, and their expression is activated through immune system sentinel receptors, such as pattern recognition receptors. Despite the similar function of type I and III on antiviral infections, it is the viral tropism that dictates the relative contribution of each IFN (9). Moreover, whereas almost all nucleated cells respond to type I IFN, type III IFNs response is restricted to tissues with a high risk of viral exposure and infection, as the mucosal surfaces. The role of type II IFN in promoting host immune response to microorganisms is similarly well documented. Notably, it is also known to play a pivotal function on cancer immune surveillance, stimulating antitumor immunity and promoting tumor recognition and elimination (10–16).

This review focuses on type II IFN signaling, cellular functions, and directed therapies and was encouraged by novel findings revealing regulatory mechanisms of IFN-γ and its prognostic as well as therapeutic potential. In fact, since Wheelock who reported that IFN-γ inhibited viral replication in 1965 (17), it took around 30 years to envisage this cytokine as a target of antitumor immunity (18).

Interferon-gamma is a homodimer formed by the non-covalent association of two 17 kDa polypeptide subunits. During synthesis, after multiple N-glycosylation, both subunits bind in an antiparallel manner, constituting a mature 50 kDa molecule (19, 20). Notably, the IFN-γ symmetry suggests that a single molecule can bind simultaneously to two receptors, amplifying the underlying responses. Cellular responses induced by IFN-γ may also involve cross-communication with IFN-α/β receptors, amplifying IFN-γ signaling and its effects (21, 22).

Interferon-gamma is secreted predominantly by activated lymphocytes such as CD4 T helper type 1 (Th1) cells and CD8 cytotoxic T cells (23–26), γδ T cells (27–33), and natural killer (NK) cells (34, 35) and, to a less extent, by natural killer T cells (NKT), B cells (36–39), and professional antigen-presenting cells (APCs) (40–42). Its expression is induced by mitogens and cytokines, such as IL-12 (43, 44), IL-15 (45), IL-18 (46, 47), and type I IFN (48, 49). IFN-γ pleiotropic functions are mediated by cell-specific expression of hundreds of IFN-γ-regulated genes that encompass inflammatory signaling molecules, apoptosis and cell cycle regulators, and transcriptional activators (50). Autocrine IFN-γ produced by APCs can act locally and contribute to sustain self and neighbor cell activation (51–53), crucial for early control of pathogen spreading, while T lymphocytes are the major paracrine source of IFN-γ in adaptive immunity. Under physiological conditions, the constitutive expression of type I and II IFNs is tightly controlled, remaining localized to tissues, without systemic effects (54–56). For instance, constitutive expression of endogenous IFN-γ contributes to the homeostasis of immune cell functions (57), maintenance of the hematopoietic stem cell niche (58), and bone formation (59). Combination approaches to boost innate immune activation have been explored to converge onto IFN pathways. However, IFN-γ-related signaling can also have suppressive immunoregulatory effects on antiviral (60, 61), autoimmune (62, 63), as well as on antitumor responses (64, 65). Unveiling cellular targets of IFN-γ is critically important for its therapeutic application, to predict patient responses, particularly in cancers where this cytokine can exert protumorigenic effects. Therefore, the cellular and molecular effects of IFN-γ, with particular emphasis on its dual role on tumor immunity and how to overcome its limitations, will be the major focus of this review.

CANONICAL SIGNALING AND REGULATORY MECHANISMS

The IFN-γ Receptor

The IFN-γ receptor is composed of two ligand-binding IFNγR1 chains associated with two signal-transducing IFNγR2 chains, which are responsible for connecting to the cytoplasmic transduction machinery (see **Figure 1**). The *IFNGR1* and *IFNGR2* are localized in chromosome 6 and 21, respectively, and their

expression differs significantly. While IFNγR1 is constitutively expressed at moderate levels on the surface of almost all cells, IFNγR2 is constitutively expressed at low levels, and its expression is tightly regulated, according to the state of cellular differentiation or activation (66). For example, CD4 T helper cell subsets differ in their ability to respond to IFN-γ (67, 68). Remarkably, IFN-γ activates the signal transducer and activator of transcription (STAT) 1 that maintains the expression of T-bet, the master transcription factor that controls IFN-γ expression in T cells (69). This signaling constitutes a positive feedback loop that maximizes Th1 immunity (70–72). Notably, Th1 cells are more resistant to the antiproliferative effects of IFN-γ than Th2 cells. This is likely due to lower levels of expression of the IFNγR2 subunit that allows Th1 cells to continue to proliferate during IFN-γ signaling. By contrast, Th2 cells that do not produce IFN-γ express higher levels of the IFNγR2 subunit, rendering them particularly susceptible to the presence of IFN-γ that inhibits their proliferation (67, 68, 73). Nevertheless, IFNγR2 downregulation may be also induced in Th2 cells when they are exposed to IFN-γ (68). Thus, IFN-γ appears to regulate the expression of its own receptor on specific cell types, representing a regulatory mechanism of cellular desensitization in response to cytokines present at the local microenvironment. As a result, IFNγR2 expression can be a limiting factor in IFN-γ responsiveness and functional outcome that can dictate the Th1–Th2 phenotype switch and modulate the subsequent immune response.

JAK/STAT Signaling Pathway

The biological effects of IFN-γ are elicited through activation of intracellular molecular signaling networks, mainly *via* the JAK/STAT pathway, which modulates the transcription of hundreds of genes and mediates diverse biological responses (50, 74–76). Upon IFN-γ binding, the intracellular domains of IFNγR2 oligomerize and transphosphorylate with IFNγR1, activating

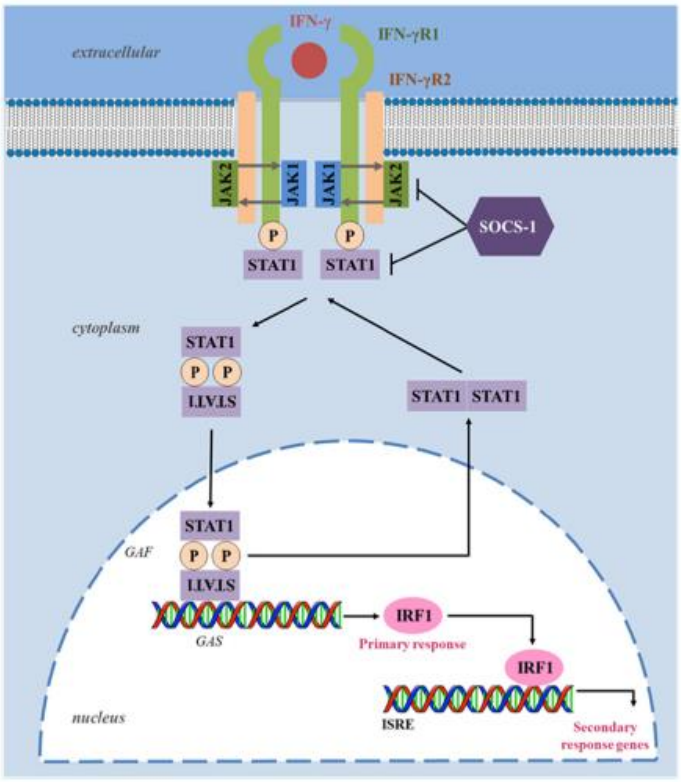

FIGURE 1 | Interferon-gamma (IFN-γ) canonical signaling pathway. Upon ligand binding, IFNγR1 and IFNγR2 oligomerize and transphosphorylate, activating Janus activated kinase (JAK) 1 and JAK2. These, in turn, phosphorylate IFNγR1, creating a docking site for the signal transducer and activator of transcription (STAT) 1. Phosphorylated STAT1 homodimerizes in an antiparallel configuration, forming a complex gamma-activated factor (GAF), which translocates to the nucleus and binds to gamma-activated site (GAS), located at the promoters of primary response genes, increasing their transcription. Upon induction, transcription factor interferon-regulatory factor 1 (IRF1) binds to interferon-stimulated response element (ISRE) and enhances the transcription of several secondary response genes responsible for several immunomodulatory functions. Suppressor of cytokine signaling (SOCS) proteins negatively regulate the IFN-γ pathway by inhibiting JAKs and STAT1 phosphorylation. Through dephosphorylation and deacetylation, the configuration of STAT1 homodimers reverts to parallel, triggering their exit from the nucleus.

the downstream signaling components, JAK1 and JAK2. The activated JAKs phosphorylate the intracellular domain of the receptor (tyrosine 440 on human IFNγR1), creating binding sites for STAT1 (77). STAT1 is then phosphorylated in the C-terminus on tyrosine Y701 residues by JAK, resulting in the formation of STAT1 homodimers complexes, known as gamma-activated factors (GAFs), which translocate to the nucleus and regulate gene expression through binding to gamma-activated site (GAS) elements in the promoters of interferon-stimulated genes (ISGs) (78). One of the major primary response genes induced by STAT1 signaling is the transcription factor interferon-regulatory factor 1 (IRF1), a member of the IFN regulatory transcription factor family (79). IRF1 functions as a transcription activator of interferon-stimulated response elements (ISRE), leading to the transcription of a large number of secondary response genes (**Figure 1**). For instance in breast cancer cells, a genome-wide identification of IFN-γ-induced IRF1 activation reveals over 17,000 binding sites, with "apoptosis" or "cell death" as the most enriched target processes underlying the direct tumoricidal property of the cytokine (80). However, tumor cells also develop resistance to IFN-γ through differential IRF1 responsiveness, pointing out that the JAK/STAT signaling pathway needs to be tightly regulated to avoid detrimental consequences of excessive stimulation and highlighting its role on immune responses and tumorigenesis (81). STAT1 targets of the IFN-γ-mediated signaling also include the SMAD family member 7 (SMAD7), and proteins involved in cell cycle regulation, such as c-Myc and the cyclin-dependent kinase inhibitor 1A (82–84).

The JAK/STAT signaling pathway is regulated at several levels by positive and negative mechanisms. In particular, deregulation or inhibition of the JAK/STAT pathway leads to lowered immunity and is often associated with increased tumorigenesis (85, 86) or metastatic dissemination (87). STATs are also involved in the development and function of the immune system and play a role in maintaining tumor surveillance [reviewed in Ref. (88)]. STAT1, as a tumor suppressor, is deducted for its expression in tumor cells, modulates their immunological status and consequently their response to antitumor immune responses. Indeed, STAT1-deficient tumor cells were more susceptible to NK cells while STAT1-proficient tumor cells were more sensitive to $CD8^+$ T cells (89). In the same way, STAT1-deficient mice that are impaired in Th1 cell polarization, exhibited reduced IFN-γ expression and compromised cytolytic and NK lytic activity, failing to control tumor growth in contrast with wild-type mice (90). In addition, cell-autonomous tumor-suppressor functions of STAT1 have also been reported in breast cancer (91). However, there is growing evidence that STAT1 also acts as a tumor promoter (92–94) since it can enhance resistance to chemotherapeutic agents and radiation in carcinoma (95). Importantly, STAT1 also participates in the signaling from different cytokines, including IL-21, IL-27, and IL-35. These cytokines have been proposed to limit antitumor immunity in specific cellular, molecular, and microenvironmental contexts (96–101). Thus, STAT1 phosphorylation reflects not only the threshold and magnitude of IFN-γ response but also of other immune mediators, highlighting the importance of the regulation of STAT1 phosphorylation. One of the most important negative regulators of the JAK/STAT signaling pathway is the suppressor of cytokine signaling (SOCS) proteins, which expression is increased in response to IFN-γ signaling through IRF1 (102, 103). SOCS blocks the activity of JAKs by a negative feedback loop, but also regulates other cytokines downstream signaling. SH2 domains in SOCS proteins directly bind to phosphorylated tyrosine residues of activated JAKs, blocking the recruitment of signal transducer adaptors, such as STATs, and JAK activity (102). Furthermore, SOCS promote interactions that lead to ubiquitination and proteasome degradation of components of the JAK/STAT signaling (104, 105). SOCS1 even prevents regulatory T (Treg) cells from producing IFN-γ by suppression of STAT1, avoiding the conversion of Treg cells into effector cells (106). In addition, SOCS2-deficient mice showed a reduction in lung metastases and an increase in survival following melanoma challenge (107).

Alternatively, the transcriptional activity of STAT1 can be positively regulated by other signaling cascades triggered by IFN-γ binding, such as the mitogen-activated protein kinase pathway, protein kinase C, and PI3K/AKT, which phosphorylate STAT1 in its transactivation domain (108). Adding to the complexity, under certain circumstances, IFN-γ also can activate STAT1-independent pathways through other transcription factors, namely STAT3 (109), STAT5 (110), nuclear factor-kappa B (NF-κB) (111), and activator protein 1 (112). In conclusion, the primary response of IFN-γ is mediated by GAF that acts on genes with GAS binding sequence in their promoter, while the primary response of type I IFNs is mediated by ISGF3 (STAT1/STAT2/IRF9 complex) that induces genes that have ISRE in their promoter. Thus, some of the ISGs are regulated by both types of IFNs, whereas others are selectively regulated by each type of IFN, consequently potentiating the diversity of biological responses.

BIOLOGICAL FUNCTIONS

IFN-γ Actions on Immune Cells

Interferon-gamma signaling pathway coordinates several biological responses, primarily involved in host defense and immune surveillance but also in the establishment of adaptive immunity (**Figure 2**) and in the regulation of inflammation, apoptosis and cell cycle. One of the first described biological effects of IFNs was the upregulation of the major histocompatibility complex (MHC) molecules (113, 114) as well as the upregulation of the whole MHC I and II antigen processing and presentation machinery including transporter associated with antigen processing (TAP) 1/2, invariant chain, and the expression and activity of the proteasome (115–122). Furthermore, in some tumor types, such as multiple myeloma and melanoma cells, IFN-γ can also upregulate the MHC class II transactivator (CIITA) that leads to MHC class II expression (123, 124). Thus, IFN-γ initiates an immune-antigenic exposure program in the target cells, and this ensures the rapid recognition of stressed tissues. IFN-γ is a major product of Th1-mediated immune response and orchestrates Th1 effector mechanisms, as further activation of innate immunity (macrophages and NK cells) in a positive feedback loop. Upregulation of cell surface MHC class I by IFN-γ is crucial for host response to intracellular pathogens and tumor cells, due to cytotoxic T cell activation, promoting cell-mediated immunity.

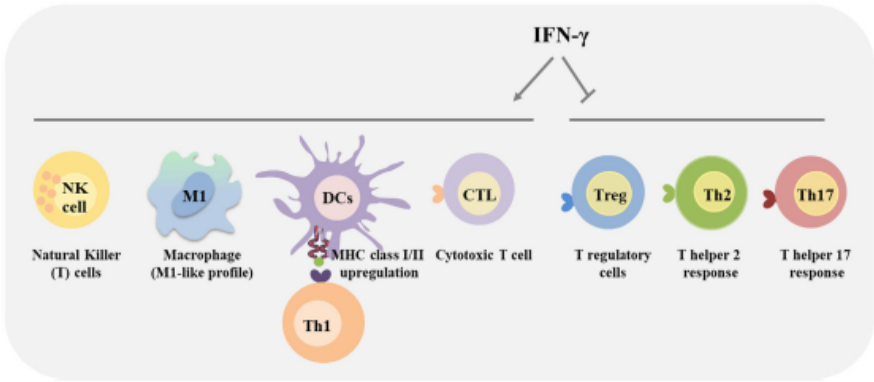

FIGURE 2 | Immunomodulatory effects of interferon-gamma (IFN-γ). IFN-γ produced by immune cells affects the behavior of distinct immune cells within the tumor microenvironment. Specifically, IFN-γ plays a major role in activating anticancer immunity, by promoting the activity of CD4 T helper type 1 cells, CD8 cytotoxic T lymphocyte (CTL), natural killer (NK) cells, dendritic cells (DCs), and macrophages, promoting the antigen presentation. Additionally, IFN-γ activates macrophages towards a more pro-inflammatory and tumoricidal phenotype (M1-like). Alternatively, IFN-γ inhibits regulatory T (Treg) cells, Th2 and Th17 differentiation and functions.

IFN-γ directly acts as a cytotoxic CD8 T cell differentiation signal, and it is essential for the induction of cytotoxic T cell precursor proliferation (125, 126). IFN-γ also upregulates cell surface MHC class II on APCs, thus promoting peptide-specific activation of CD4 T cells (25, 127–129). In addition, IFN-γ activates macrophages toward a pro-inflammatory profile, exhibiting an increased phagocytic ability as well as enhanced microbial killing activity (130). In fact, IFN-γ was initially shown to induce "classical" activation of macrophages and polarization toward a tumoricidal phenotype (131). Interestingly, the original name of IFN-γ was macrophage activation factor (132, 133). IFN-γ controls specific gene expression programs involving more than 290 genes related to cytokine and chemokine receptors, cell activation markers, cellular adhesion proteins, MHC proteins, proteasome formation, protein turnover, and signaling mediators and regulators (134). The ability of IFN-γ to induce tumor cell killing includes the activation of the NADPH-dependent phagocyte oxidase system, nitric oxide production, tryptophan depletion and upregulation of lysosomal enzymes (121, 135, 136). These events result in recruitment of effector cells to help in the inflammation resolution process (137, 138). In addition, as a major cytokine of Th1 cells, IFN-γ maintains Th1 lineage commitment through a positive feedback loop that stabilizes the Th cell phenotype (72, 139–141) and cross-inhibits the differentiation to other Th cell subsets (**Figure 2**). Indeed, IFN-γ inhibits Th2 cell differentiation (142, 143) and consequently IL-4 production. This regulation involves the inhibition of the IL-4/STAT6 pathway, required for Th2 cell differentiation, and it is mediated at least by IFN-γ-induced SOCS1 that inhibits IL-4R signaling (144, 145). Furthermore, IFN-γ-induced T-bet inhibits Th2 cell differentiation by directly interfering with the activity of Th2 cell-specific transcription factor, GATA-3 (146). Höfer and colleagues, using mathematical models, proposed that IL-4 also acts to propagate Th2 cell differentiation (147). A high IL-4 level promotes increased GATA-3 expression that further enhances GATA-3 transcriptional imprinting for Th2 differentiation (147, 148). This model proposed that high expression state of GATA-3 can be suppressed by strong inhibition of autoactivation, as observed in the presence of Th1-polarizing conditions (147, 149). IFN-γ was also described to downregulate the IL-4-inducible gene expression (150). The cross-regulation of Th1 and Th2 cells was also demonstrated in STAT6-deficient mice, which lack Th2 phenotype and associated immune responses. These animals displayed augmented tumor-specific IFN-γ production and cytotoxic T cell activity and, consequently rejected the tumor cell line that grew progressively in the wild-type control (151).

Interferon-gamma produced by Th1 cells also counteracts Th17 cell development and their effector functions (152–154). Several mechanisms can be considered as the inhibition of molecules involved in the Th17 differentiation (155, 156), the inhibition of STAT3 by STAT1 (157) and recently, T-bet was demonstrated to prevent differentiation of Th precursors into Th17 cells by blocking the expression of the Th17 cell lineage-specific transcription factor, RORγt (158). Furthermore, IFN-γ also exerts regulatory functions to limit tissue damage associated with inflammation (63, 159–162) (**Figure 2**). IFN-γ has been classically considered as a pro-inflammatory cytokine, involved in the regulation of anti-inflammatory responses, by antagonizing the IL-10 (157, 163) and TGF-beta (164) signaling pathways. Consequently, IFN-γ inhibits Treg cell differentiation and functions (165, 166). However, in some chronic inflammation conditions, IFN-γ plays a crucial role in attenuating tissue destruction. In this case, IFN-γ might be protective (62, 167) by promoting the number and function of Treg cells (168–170). In addition, IFN-γ production by Treg cells themselves was shown to be a key feature of the Treg cells that are capable of dampening Th1 cell responses (171–174). Thus, IFN-γ dictates the differentiation of specialized Foxp3$^+$T-bet$^+$ Treg cells that selectively suppress Th1 cells, and constitute a negative feedback loop to minimize the detrimental effect of IFN-γ. IFN-γ also promotes the differentiation of

myeloid-derived suppressor cells (MDSCs) that restrain overactivation of effector T cells, maintaining tissue homeostasis (175, 176). Other regulatory mechanisms involving IFN-γ signaling that dampen the magnitude of the immune response have been reported, as the induction of indoleamine 2,3-dioxygenase (IDO) by Treg cells, monocytes and stromal cells (177–180), and of the programmed cell death 1 (PD-1) ligand (PD-L1) on immune and transformed cells, inhibiting T cell responses (181–183).

IFN-γ Actions on Transformed Cells and on the Tumor Microenvironment

Interferon-gamma is involved in antiproliferative (18), antiangiogenic (184) and pro-apoptotic effects established against neoplastic cells. How IFN-γ induces the signaling pathways initiating and propagating the apoptotic cascade remains to be elucidated. The level of complexity is demonstrated by the fact that the mechanism might depend on the tumor cells themselves. For example, while in a glioblastoma cell line the induction of apoptosis was due to suppression of the PI3K/AKT pathway, in another glioblastoma cell line apoptosis occurred independently of the PI3K/AKT pathway but required NF-κB (185). It was also shown that IFN-γ induces apoptosis of human pancreatic carcinoma cells in a caspase-1-dependent manner (186). A review covered in detail the mechanism of induction of programmed cell death (187). So far, the known biological functions of IFN-γ indicate that, although it can act as a potent inducer of antitumor immunity, it actually has a dual role and may also favor tumor immune evasion.

IFN-γ IN CANCER

The first reports pointing to the relevance of IFN-γ in antitumor immunity came from studies with the fibrosarcoma (Meth A) cell line, refractory to IFN-γ signaling, since it lacks the expression of the IFNγR1 subunit. IFN-γ-insensitive Meth A cells displayed enhanced tumorigenicity compared with control cells and were not rejected in syngeneic tumor mice models, suggesting that IFN-γ plays an important role in tumor cell elimination (18). This finding was further supported by experiments using 129/SV IFN-γ insensitive mice, lacking the IFNγR1 subunit or STAT1, which developed 3-methylcholanthrene (MCA)-induced sarcomas more rapidly and more frequently than their wild-type counterparts (12). Similarly, these IFN-γ-insensitive mice lacking the tumor-suppressor protein p53 formed spontaneous tumors more rapidly than IFN-γ-sensitive p53-deficient mouse (12). In addition, C57BL/6 mice that lack the gene encoding IFN-γ also displayed higher susceptibility to experimental (B6, RM-1 prostate carcinoma) and spontaneous (BALB/c, DA3 mammary carcinoma) models of primary and metastatic tumors (13, 14). Notably, further studies described that IFN-γ may cooperate with other molecules to prevent tumor formation. Mice deficient in both granulocyte/macrophage colony-stimulating factor (GM-CSF) and IFN-γ developed lymphoma and non-lymphoid solid tumors at a higher rate than did mice deficient in GM-CSF or IFN-γ alone (15). Additional studies revealed that mice insensitive to IFN-γ, or that lack the recombination activating gene (RAG) protein (failing to produce mature B and T lymphocytes), or that lack both, showed similar incidence of MCA-induced sarcomas, suggesting that the T cell–IFN-γ axis is involved in immune surveillance (10).

The role of IFN-γ on cancer immunoediting emerged from studies assessing the immunogenicity of tumors from immunocompetent versus immunodeficient mice. Kaplan et al. showed that MCA-induced sarcoma cells from IFNγR1-deficient mice (unresponsive to IFN-γ signaling) grow as aggressively in immunocompetent as in IFNγR1-deficient mice. However, when IFN-γ responsiveness was conferred on the tumor cells by introducing the IFNγR1 subunit, they became more immunogenic and were rejected through a T cell-dependent manner (12). This constitutes the first demonstration that IFN-γ sensitivity of the tumor is fundamental for an efficient antitumor response. Other studies revealed that wild-type hosts rejected 40% of MCA-induced sarcomas derived from RAG2-deficient mice, showing that these tumors were more immunogenic than those from wild-type mice (10). In addition, human tumors were evaluated for their ability to upregulate MHC I expression in response to IFN-γ stimulation. These studies revealed that 33% of 33 melanoma tumor cell lines showed a reduction in IFN-γ sensitivity while 4 of 17 lung adenocarcinoma cell lines were totally unresponsive to IFN-γ (12). This lack of response resulted from cellular defects on IFNγR1 and of JAK proteins and may explain the ability of many tumor cells to evade the immune response. Recently, JAK1/2 deficiency was demonstrated to protect melanoma cells from antitumor IFN-γ activity and results in T-cell-resistant melanoma lesions (188). Others reported the lack of STAT1 in melanoma cell lines and in some chronic myeloid leukemia cells (189). Furthermore, DNA methylation that selectively represses CIITA, in colorectal and gastric cancer cell lines, was associated with the absence of IFN-γ-induced HLA-DR, suggesting that this epigenetic alteration of CIITA enables some gastrointestinal cancer cells to evade the immune system (190). Concomitantly, epigenetic alterations repressing *MHC2TA* were described in T cell leukemias, B cell lymphomas, and in several cancer cells, such as small cell lung cancer and neuroblastoma cells that were unable to express MHC II upon IFN-γ stimulation (191–194). Consistently, IFN-γ upregulates CIITA expression on multiple myeloma and melanoma cells increasing their MHC II expression (123, 124). These findings indicate that IFN-γ acts on tumor cells, enhancing their recognition by CD8 T cells as well as by CD4 T cells, and unveiling a key role in the promotion of tumor immunogenicity. Altogether, these works pave the way for the elaboration of the stepping-stone concept of immunoediting promoted by IFN-γ (195, 196).

IFN-γ-Mediated Mechanisms Underlying Antitumorigenic Effects

As described earlier, the mechanisms by which IFN-γ exerts its antitumor effects depend on multiple processes. IFN-γ is described as an antiproliferative agent that regulates the expression of cyclin-dependent kinase inhibitor 1 (p21) through STAT1 activation in tumor cells (84, 197). Moreover, IFN-γ is able to promote tumor cells apoptosis by upregulating the expression of caspase-1, -3, -8 (198, 199) and by enhancing the secretion of FAS and FAS ligand (200) and TNF-related apoptosis-inducing

ligand (201, 202). Recent studies showed that IFN-γ also induces its tumoricidal effects through a form of regulated necrotic death (also named as necroptosis) that relies on the activity of the serine–threonine kinase RIP1 (203). Importantly, IFN-γ is also involved in the inhibition of angiogenesis, impairing the proliferation and survival of endothelial cells, inducing ischemia in the tumor stroma (184, 204, 205). In particular, IFNγR is expressed on blood endothelial cells and engagement of the receptor results in blood vessel destruction and necrosis, an important mechanism that leads to tumor rejection (206).

Considering the effect of IFN-γ on the host immune cells present at the tumor microenvironment, major efforts have been made for the development and establishment of combined clinical therapeutic applications (90, 151, 207). IFN-γ is critical for T cell, NK and NKT cell trafficking into the tumors through CXCL9, CXCL10, and CXCL11 chemokine induction (208, 209). Accordingly, T cells fail to migrate to tumor site in IFNγ-deficient mice (65). In commitment, dipeptidylpeptidase 4 inhibition, a protease that inactivates these chemokines, enhanced tumor rejection by increasing lymphocytes trafficking into the tumor (210). Lately, galectin-3 secreted by several tumors was demonstrated to bind glycosylated IFN-γ at the tumor extracellular matrix, avoiding IFN-γ diffusion and the formation of an IFN-γ-induced chemokine gradient required for T cell recruitment and infiltration (211). In addition, CXCL10 also prevents tumor angiogenesis by blocking endothelial cell proliferation (212) and consequently a decrease in microvessel density as observed in melanoma tumor xenografts (213). Apoptosis of endothelial cells by IFNs causes restriction of blood flow within the tumor vasculature, leading to tumor shrinkage (214). This is an effect of IFN-γ, not directly targeted to the tumor cell, but to the tumor vasculature, with drastic and desirable effects on tumor growth. A recent report also showed that IFN-γ was essential for the initial priming and differentiation of cytotoxic T cells residing in the periphery of the eye, contributing to the regression of intraocular tumors (215). Supporting data from therapy models showed that IFN-γ induces *survivin* and *ifi202*, two genes involved in T cell maturation, survival, and proliferation, in tumor-specific T cells (216). Overall, these studies demonstrated the relevance of IFN-γ on T cell-mediated antitumor immunity.

Interferon-gamma is also involved in macrophages tumoricidal activity (217). This cytokine supports a CD4 T cell/macrophage effector axis which acts as immune surveillance mechanism for MHC II-negative cancer cells (25). Indeed, upon recognition of tumor antigens present in the context of MHC II by macrophages, CD4 T cells secrete IFN-γ that further activates macrophages in the tumor, leading to tumor growth inhibition (25). This collaboration between CD4 T cells and macrophages was also essential for successful cancer immune surveillance in non-solid cancers, as myeloma and B-cell lymphoma. Indeed, Th1-secreted IFN-γ was shown to trigger a cytotoxic activity of tumor-associated macrophages (TAMs) and also induces CXCL9/MIG and CXCL10/IP-10 secretion by macrophages, which may affect the tumor progression by angiogenesis inhibition (129). IFN-γ-activated macrophages also acquire a tumoricidal phenotype with the upregulation of cytotoxicity-associated markers including granzyme A/B, and NKG2D (129). In addition, in STAT6-deficient mice, that display increased levels of IFN-γ, rejection of metastatic disease after removal of the primary tumor involved the generation of pro-inflammatory macrophages, also termed M1-like macrophages, and a decrease in MSDCs that accumulated during primary tumor formation (218). Studies from $APC^{Min/+}$ mice (that are highly susceptible to spontaneous intestinal adenoma formation) lacking IFN-γ signaling showed an accumulation of TAMs, more prone towards protumoral (M2-like) polarization, and upregulation of matrix metalloproteases. These results suggest that IFN-γ unresponsiveness contributes to the creation of an anti-inflammatory microenvironment, favorable to intestinal tumorigenesis (219). The properties of IFN-γ to reverse the myeloid immunosuppressive functions were also demonstrated in protumor role of human ovarian TAMs (220) and human M2-like macrophages (221).

Importantly, IFN-γ has also a key role on IL-12 production, supporting the activity of this later cytokine in cancer immune surveillance (222–225). Indeed, exogenous IL-12 administration into fibrosarcoma-bearing mice resulted in a complete tumor regression (222). This observation was extended to primary tumorigenesis models treated with exogenous IL-12 (226, 227). Consistent with this, chimeric antigen receptor-redirected T cells engineered to produce IL-12 where found to secrete increased IFN-γ levels and to display enhanced antitumor cell activity (228–230).

Regarding the importance of IFN-γ in cancer diagnostics, IFN-γ-associated signatures have a predictive value in cancer immune phenotypes (81, 231, 232). In addition, IFN-related gene signature is a predictive marker for chemotherapy and radiotherapy efficiency for breast cancer (94) as well as to PD-1 or cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade in various types of malignancies (233–235). Consistently, immunotherapy using immune checkpoint blockers (anti-CTLA-4 and/or anti-PD-1) combined with anticancer vaccines, clearly associate inhibition of tumor growth with increased proportion of IFN-γ-producing effector T cells (236, 237). This is also verified in clinical trials, through which the anti-CTLA-4 therapy was associated with an increase of IFN-γ-producing $ICOS^+$ (inducible costimulatory) CD4 T cells and of T effector/Treg cell ratio in bladder cancer samples (238). In addition, PD-1 blockade was demonstrated to enhance T cell infiltration by promoting IFN-γ-inducible chemokines (239). In other way, it was recently shown that IFN-γ-induced Treg cell fragility (loss of suppressive function) is required for response to anti-PD-1 therapy (240).

Altogether, the versatility of IFN-γ and its fine-tuned biological effects highlight its relevance for therapeutic applications, and some clinical trials have already encouraging results. In fact, 75% of metastatic melanoma patients were non-responders to anti-CTLA-4 therapy, and this was associated with genomic defects of IFN-γ signaling genes on tumors (241). Recently, apelin receptor (*APLNR*) was described to regulate JAK/STAT signaling, modulating IFN-γ responses. Multiple loss-of-function mutations in *APLNR* were identified in patient tumors refractory to immunotherapy (242). The inclusion of IFN-γ in the first-line treatment of ovarian cancer resulted in benefit regarding progression-free survival, with acceptable toxicity (243). IFN-γ treatment also appears to be effective against bladder tumors by recruitment and activation of intratumoral

leukocytes (244). In a phase I clinical trial, which combined adoptive T cell therapy with intralesional administration of adenovirus expressing IFN-γ in metastatic melanoma, 38.5% of the patients had an overall objective response and 46% were able to control the disease (245).

IFN-γ-Mediated Mechanisms Underlying Protumorigenic Effects

It is becoming increasingly clear that IFN-γ can exert certain effects supporting tumorigenesis. Immune evasion can operate through tumor cells losing responsive to IFN-γ signaling to avoid its antiproliferative, pro-apoptotic, and immunoregulatory actions. This has been demonstrated with the tumor cells losing the receptor for IFN-γ or a component of JAK/STAT signaling (12, 18). In addition, constitutive activation of inhibitory molecules of this pathway, as SOCS1 and SOCS3, limits the actions of IFNs on human melanoma cells (246) and favors the activation of alternative signaling pathways, as STAT3, which is associated with tumor progression (247). These evidences suggest that tumor cells develop IFN-γ-dependent strategies to evade the immune system, leading to the emergence of very aggressive tumors, which are on the basis of immunoediting. In 2011, Zaidi and Merlino proposed that IFN-γ actions might play a physiological role in protecting cells from damage in a setting of tissue remodeling and repair, while on cells harboring oncogenic mutations, the same mechanisms may prevent cell destruction and allow complete transformation (248). Consistent with this, NF-κB in tumor cells was shown to act as a protective mechanism against IFN-γ-induced necroptosis (203).

Indeed, there are significant evidences that tumor cells can take the advantage of IFN-γ as an inducer of anti-inflammatory responses and protumor effects. The first report of the negative potential effects was in 1987 by Taniguchi and colleagues who proposed that IFN-γ changes the metastatic ability of the B16 melanoma cells in a cell-autonomous manner (249). Data from experiments using the CT26 colon carcinoma model showed that IFN-γ promotes tumor escape through the downregulation of the endogenous tumor antigen gp70 (250). IFN-γ expression by human melanoma samples was associated with enhanced expression of MHC class II molecules and the acquisition of a more aggressive phenotype (251, 252).

One of the principal mechanisms of tumor immune escape is the suppression of cytotoxic T cells and of NK cell-mediated immune responses. Brody and colleagues showed that IFN-γ upregulates IDO in melanoma cells and recruits Treg cells to avoid immune recognition (253). Curiously, IFN-γ induced IDO competence on human monocyte-derived DCs but had no effect on pro-inflammatory cytokine release, suggesting that IFN-γ triggers IDO activity and pro-inflammatory cytokine release as distinct cellular programs. In addition, IDO-competent DCs induced regulatory activity on allogeneic T cells (179). IFN-γ was also described to be involved in the accumulation of MDSCs in inflamed liver, which leads to T cell suppression (254). MDSCs producing nitric oxide decreased IFN-γ responsiveness of immune cells, such as T and NK cells (255).

One important aspect is the ability of IFN-γ to induce PD-L1 expression in cancer, stromal and myeloid cells to impair effector tumor immunity (181). Abiko and colleagues demonstrated that the contact between tumor cells and CD8 T cells is necessary for the induction of PD-L1, underlying the importance of paracrine exposure to IFN-γ (256). Recent reports suggest that loss of IFN-γ pathway genes, such as JAK1 and JAK2, is associated with resistance to anti-PD-1 therapy (257, 258). Prolonged IFN-γ signaling in tumors was also shown to coordinate PD-L1-dependent and PD-L1-independent resistance to immune checkpoint blockade and to other therapeutic combinations, such as radiation and anti-CTLA-4, through a multigenic resistance program (259). In addition, other inhibitory pathways are reinforced by IFN-γ, including CTLA-4 and CD86/CD80 interaction (260).

Interferon-gamma was used in clinical trials for melanoma but no significant improvement for patients was observed (261–264). In fact, IFN-γ treatment had no contribution to the outcomes of patients with metastatic renal cell carcinomas (265), leukemia (266), pancreatic carcinoma (267), breast cancer (268), or into the postoperative surgical therapy for colon cancer (269). Furthermore, a phase 3 trial of IFN-γ plus standard treatment with carboplatin/paclitaxel versus carboplatin or paclitaxel alone, for treated advanced ovarian tumors, was early terminated due to a higher incidence of serious hematological toxicities in patients receiving combined therapy compared with chemotherapy alone (270). The failed attempts to treat cancer patients with exogenous IFN-γ raised several concerns: the absence of tumor immunogenicity, the lack of IFN-γ-signaling components, the upregulation of IFN-γ signaling inhibitors, the immunosuppressive tumor microenvironment, the lack of effector T cells, or presence of anergic T cells and, in some cases toxicity. These accumulating evidences reinforce the importance to determine the grade of patients' IFN-γ-responsiveness. For example, in cases with low IFN-γ actions, active immunization either *via* IFN-γ treatment or *via* adjuvants of the immune system, as toll-like receptor ligands, should be considered, as demonstrated recently by using bacterial outer membrane vesicles that eradicate established tumors in an IFN-γ-dependent mechanism (271). The combination with radio- and chemotherapy is expected to be useful through immunogenic cell death that also elicits the innate immune system. Promising results were obtained with combination of low-dose 5-fluorouracil with recombinant interferon-gamma (IFN-γ) in patients with advanced hepatocellular carcinoma (272). In cases with high levels of IFN-γ signaling, the therapy with anti-PD-1/anti-PD-L1 is expected to be important.

Overall, these findings indicate that the local immune microenvironment of tumors is complex and variable and that for an effective therapy it is essential to evaluate, individually, the immune profile of patients or immune contexture [reviewed in Ref. (232, 273)], taking into account that it may evolve and modify throughout the anticancer therapy (**Figure 3**).

IFN-γ IN THERAPY—WHERE ARE WE AND WHERE ARE WE GOING?

Interferon-gamma therapy has ensued in clinical applications approved by the Food and Drug Administration in the treatment of chronic granulomatous disease, in 1999 and severe

malignant osteopetrosis, in 2000. Despite the promising therapeutic applications of IFN-γ in several settings, its limited success in cancer-immunotherapy trials might be due to cancer cell unresponsiveness to this cytokine, the failure to deliver it locally or with the adequate periodicity to achieve a therapeutic effect. Moreover, IFN-γ clinical use has also been restricted due to several limitations inherent to its molecular properties. Essentially, these include stability problems, such as acid degradation, and also the tendency to aggregate irreversibly under mild denaturing conditions, with subsequent loss of biological activity [the pharmacological aspect of IFN-γ is reviewed in Ref. (274, 275)]. Furthermore, IFN-γ is rapidly cleared from the blood when administered intravenously (276), requiring frequent re-administrations of high cytokine concentrations, to elicit an effective response at the target site, leading to systemic toxicity and side effects, such as fever, fatigue, nausea, vomiting, diarrhea, neurotoxicity, and leukopenia (277). These adverse effects are caused mainly by high serum concentration of the protein, due to an unequal distribution between body fluids and tissues (276) and, additionally, to the ubiquity of receptors which are expressed at the membrane of the majority of human cells (278, 279) and also to the existence of a circulating soluble form (which function remains elusive) (280).

These constraints in the clinical use of IFN-γ have encouraged the development of alternative delivery methods with the purpose of achieving higher therapeutic outcomes and, simultaneously, weaken its toxicity. Numerous reports have focused mainly on efficient routes of delivery rather than on systemic applications (281–287). In fact, IFN-γ is naturally produced in a paracrine manner, with local secretion and diffusion to the surrounding cells and microenvironment throughout the extracellular fluids (288). Therefore, a localized delivery of this cytokine has been

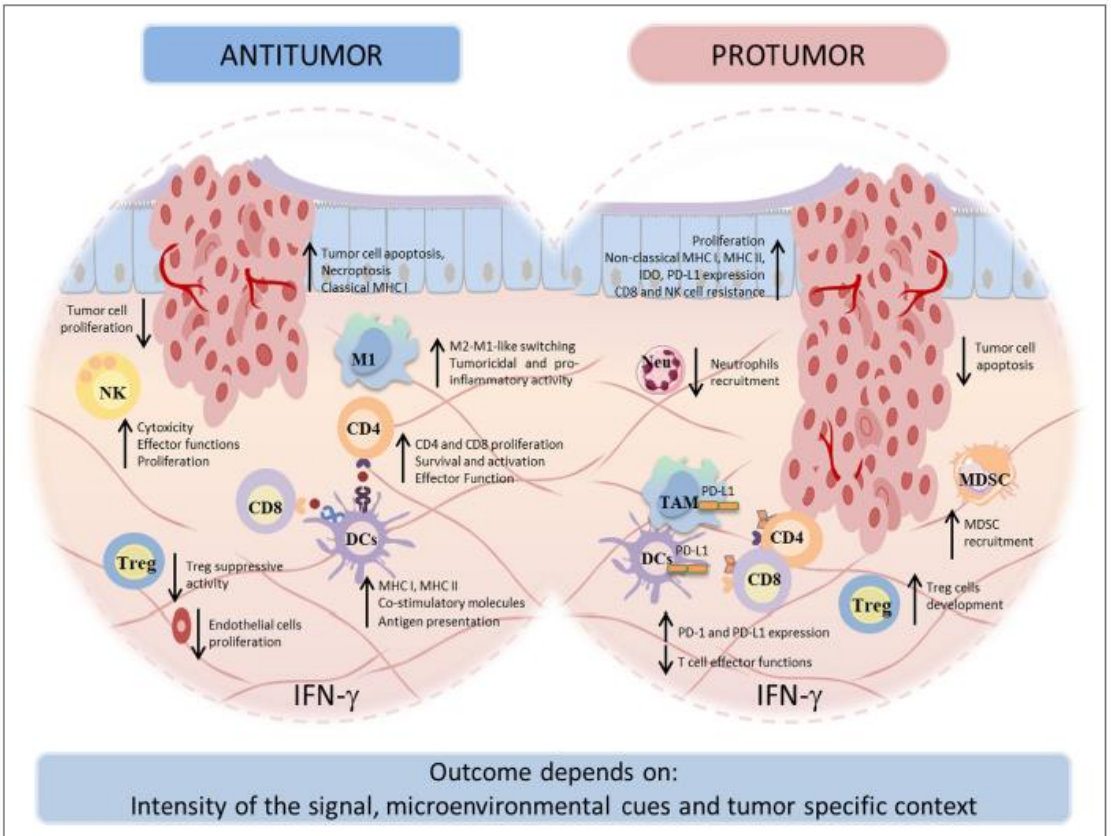

FIGURE 3 | Dual face of interferon-gamma (IFN-γ) in tumor immunity. IFN-γ can display both antitumor and protumor activities. Under both circumstances, IFN-γ influences tumor cells directly and indirectly, by activation of immune cells. The antitumor effects comprise the development, recruitment, and activation of innate immune cells as well as the activation and maintenance of effector T cells. The antitumor effects of IFN-γ result in direct inhibition of tumor proliferation, recognition, and elimination. In other way, the protumorigenic role of IFN-γ involves proliferative and antiapoptotic signals, as well as escape of the tumor cells from recognition and cytolysis by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. The broad range of IFN-γ actions depends on the context of tumor specificity, IFN-γ-signaling intensity, and other microenvironment conditions.

determined to be more appropriated in terms of therapeutic efficiency, due to its specific effect at the target site, while simultaneously intensifying the intended cytotoxic effects and immunological stimulation (289). In particular, tumors can be rejected by local IFN-γ expression, but rejection of established tumors was less efficient over time, suggesting that timing of treatment plays a critical role, for transplanted tumors became less susceptible to local IFN-γ treatment the better they are established (206). Another relevant aspect concerns the mode of administration, being it an intermittent or sustained release. Several studies concluded that a sustained release strategy is more efficient by limiting the exposure of other cells and organs to the deleterious effects of high IFN-γ concentrations (290–295). In the particular case of cancer immunotherapy, consistent findings show that a stable and high concentration at the target site is required to elicit an effective response (288, 296), prompting several attempts to promote local delivery of IFN-γ with controlled release. These include liposomes, polymer gels, biodegradable microspheres, gene therapy, and magnetic or albumin nanoparticles (285, 297–301). However, these strategies revealed unsuccessful by failing to maintain a stable and/or bioactive cytokine prior release, an inadequate release rate, a labor intensive and cost ineffective manufacture, and safety issues. Oncolytic viruses have gained interest for immunotherapy due to their ability to selectively destroy tumor cells and to their potential to stimulate antitumor immunity. Oncolytic vesicular stomatitis virus expressing IFN-γ demonstrated greater activation of DCs, higher pro-inflammatory cytokines' secretion, and reduced tumor growth in 4T1 tumor model compared with the parental virus, suggesting that specific production of the IFN-γ within the tumor microenvironment is beneficial for the antitumor immune response (302). Recently, an IFN-γ-delivery system based on chitosan/poly(γ-glutamic acid) polyelectrolyte complexes was described by our group to successfully decrease macrophage-derived stimulation of cancer cell invasion *in vitro* through the modulation of a pro-inflammatory macrophage phenotype (221). In fact, several efforts have been directed to educate APCs toward an immunostimulatory and antitumor phenotype (**Figure 4**) (303–306). In another work, a silk-based hydrogel was designed to regulate cytokine delivery for macrophages, which are actively involved in tissue remodeling and vascularization, with the aim to regulate the microenvironment of biomedical implants (307). Other potential strategy to improve the shorter half-live of IFN-γ is fusing it with antibodies, enhancing its stability in the serum and tumor target specificity and reducing toxic side effects (308). Although promising results have been achieved with some of these strategies, the desired requirements are yet to be accomplished and need further investigation/development.

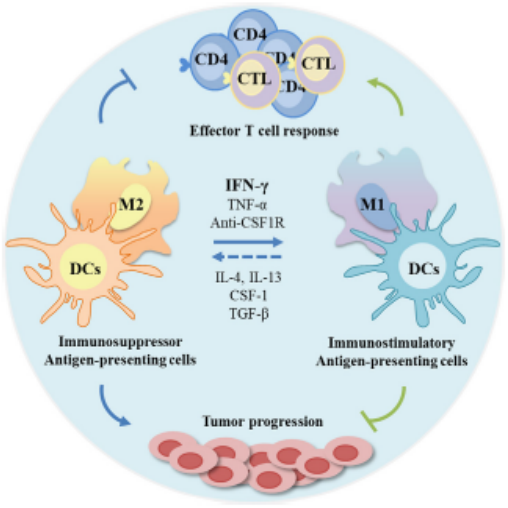

FIGURE 4 | Modulation of antigen-presenting cells (APCs) profile as anticancer therapeutic strategies. The tumor microenvironment is frequently immunosuppressive with APCs functions compromised, and consequently with poor T cell response. As APCs can be modulated by microenvironmental signals, these cells are promising targets. Interferon-gamma (IFN-γ) and other molecules can be used to re-educate tumor-associated macrophages, frequently associated with anti-inflammatory status (M2-like) toward a pro-inflammatory and antitumor profile, while stimulating regulatory dendritic cells (DCs) to an immunostimulatory profile. This stimulation can potentiate effector T cell response and inhibit tumor progression.

CONCLUDING REMARKS

Herein, we discussed the role of IFN-γ on tumor immunity and its potential therapeutic implications. On one side IFN-γ appears as a promoter of tumor immune surveillance and on the other as a supporter of tumor escape. The outcome of IFN-γ signaling depends on the tumor-specific context, the magnitude of the signal, and the microenvironmental cues. Nevertheless, IFN-γ or IFN-γ inducers remain promising agents to include in combined therapies against cancer. We believe that the effectiveness of future IFN-γ-based therapies will involve the development of systems to deliver the appropriate amount of cytokine to target cells, minimizing its side effects. In addition, these strategies would profit from the combination with conventional treatments and with anti-PD-L1 and anti-CTLA-4 therapies to overcome the regulatory effects of IFN-γ. Another important issue is to consider a personalized approach, which takes into account the patient responsiveness to IFN-γ, by using predictive biomarkers, as IFNγR2, SOCS, *APLNR*, STAT1, or STAT3. Thus, a comprehensive understanding of the complex and variable tumor microenvironment, as well as a deeper evaluation of the immune, vascular and stromal profile, will be necessary for the stratification of cancer patients and for the establishment of efficient personalized therapies.

AUTHOR CONTRIBUTIONS

FC performed the initial draft, written the manuscript, and designed **Figures 2–4**. AC written a part of Section "IFN-γ IN Therapy—Where Are We and Where Are We Going?" and performed **Figure 1**. RG, KS, and MO critically revised the manuscript, reorganized ideas, and approved the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER III

General aims

Myeloid cells, namely tumor-associated macrophages (TAMs), are considered one of the major dictators of immunosuppression, cancer immune escape and tumor progression, in several types of cancer. Generally, they are associated with poor prognosis and more recently, they were associated with resistance to chemo- and radiotherapy. Taking in consideration that these cells are phenotypically and functionally plastic, they appear as attractive targets for anticancer therapy. The overall objective of this thesis was precisely to develop a new therapeutic strategy, based on the immunostimulatory properties of chitosan (Ch) and of poly-γ-glutamic acid (γ-PGA), to modulate myeloid cells present at the immunosuppressive tumor microenvironment and to counteract tumor progression.

Accordingly, the following specific objectives were established:

1. Characterize the ability of Ch/γ-PGA nanoparticles to modulate in vitro human macrophages and dendritic cells profile and their associated functions

Several studies have addressed the impact of chitosan on immune cells profile, probably due to its immunogenicity and antitumor properties, while γ-PGA is being studied for its drug delivery potential, which make them appealing for biomedical applications. Considering that at the tumor microenvironment TAMs are phenotypically and functionally similar to M2 macrophages and that dendritic cells (DCs) frequently present an immature phenotype, the ability of Ch/γ-PGA nanoparticles to modulate human IL-10-differentiated macrophages, as well as immature DCs, into a pro-inflammatory profile was evaluated. Therefore, IL-10-stimulated macrophages and DCs treated with Ch/γ-PGA nanoparticles were characterized in terms of their viability, their ability to internalize Ch/γ-PGA nanoparticles, expression of specific cell surface markers and pro- and anti-inflammatory cytokines secretion. Furthermore, the effect of Ch/γ-PGA NPs-educated macrophages and DCs on T cell proliferation and activation, and on colorectal cancer cell invasion was also evaluated. These results are included in *Chapter IV.*

2. Evaluate the potential of immunostimulatory Ch/γ-PGA nanoparticles to be used in combination with radiotherapy

The concept of combinatorial therapies appeared as an attempt to avoid drug resistance, frequently observed in cancer patients. Currently, chemo- and radiotherapy are being combined with immunotherapeutic agents in clinical trials. Considering the immunostimulatory potential of Ch/γ-PGA nanoparticles, we decided to evaluate their impact on tumor progression, when used in combination with fractionated radiotherapy, in

an immunocompetent breast tumor model. For that, BALB/c mice were orthotopically injected with the syngeneic 4T1 cell line and, after seven days, animals were irradiated at the tumor nest with fractionated ionizing radiation doses (2x5Gy), following Ch/γ-PGA nanoparticles administration three times/week. The impact of such combinatorial treatment on primary tumor growth and lung metastasis formation was evaluated. These results are integrated within *Chapter V.*

3. Evaluate the immunostimulatory ability of Ch/γ-PGA nanoparticles to be used in combination with interferon-gamma treatment

Interferon-gamma (IFN-γ) appears as a crucial mediator of antitumor immunity, being associated with improved therapeutic response to immunotherapy in melanoma patients. Despite, more recently, some protumoral actions have been associated with IFN-γ, such activity seems to be dependent of the tumor microenvironment functional state. Furthermore, IFN-γ and Toll-like receptor (TLR) ligands have been described to exert a synergistic effect in antitumor profile induction. Here, we addressed the potential of Ch/γ-PGA nanoparticles, comprised by elements known to activate TLRs, to synergize with IFN-γ in the induction of antitumor immunity. Therefore, a syngeneic orthotopic 4T1 breast tumor model was treated locally with Ch/γ-PGA nanoparticles and soluble IFN-γ three times/week during 2 weeks. The impact of this combinatorial strategy on primary tumor growth and lung metastasis formation was assessed. These results are included within *Chapter VI.*

4. Design of innovative anticancer therapeutic strategy by using Ch/γ-PGA nanoparticles to incorporate IFN-γ

Based on the promising immunostimulatory synergistic effects of Ch/γ-PGA nanoparticles and IFN-γ concomitant administration, a new therapeutic strategy was aimed. Considering the IFN-γ stability problems, we proposed Ch/γ-PGA nanoparticles as a suitable drug delivery system for this cytokine. Therefore, Ch/γ-PGA/IFN-γ nanoparticles were developed and their ability to modulate macrophage and DCs inflammatory profile and to counteract their pro-invasive ability was evaluated *in vitro*. Although a proof of concept, these results, integrated within *Chapter VII* settle the basis for the development of a novel immunomodulatory therapy.

CHAPTER IV

Pro-inflammatory chitosan/poly(γ-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity

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Pro-inflammatory chitosan/poly(γ-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity

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ABSTRACT

Anticancer immune responses depend on efficient presentation of tumor antigens and co-stimulatory signals provided by antigen-presenting cells (APCs). However, it is described that immature dendritic cells (DCs) and macrophages at the tumor site may have an immunosuppressive profile, which limits the activity of effector T cells and supports tumor progression. Therapeutic targeting of these innate immune cells, either aiming at their elimination or re-polarization towards an immunostimulatory profile, has been pointed as an attractive approach to control tumor progression. In the present work, we assessed the potential of Chitosan (Ch)/Poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) to modulate macrophages and DCs inflammatory profile and to impair their ability to promote cancer cell invasion. Interestingly, Ch/γ-PGA NPs, prepared by co-acervation method, induced an immunostimulatory DCs phenotype, enhancing the expression of the co-stimulatory molecules CD86, CD40 and HLA-DR, and the secretion of the pro-inflammatory cytokines TNF-α, IL-12p40 and IL-6. Furthermore, Ch/γ-PGA NPs re-educated IL-10-stimulated macrophages towards a pro-inflammatory profile, decreasing the expression of CD163 and promoting the secretion of IL-12p40 and TNF-α. These alterations in the immune cells phenotype promoted $CD4^+$ and $CD8^+$ T cell activation/proliferation and partially inhibited APCs' ability to induce colorectal cancer cell invasion. Overall, our findings open new perspectives on the use of Ch/γ-PGA NPs as an immunomodulatory therapy for antigen-presenting cells reprogramming, providing a new tool for anticancer therapies.

Statement of Significance

The immune system is responsible to detect and destroy abnormal cells preventing the development of cancer. However, the immunosuppressive tumor microenvironment can compromise the immune response favoring tumor progression. Thus, immune system modulation towards an immunostimulatory profile can improve anticancer therapies. This research focus on the development of chitosan/poly(γ-glutamic acid) nanoparticles (NPs) to modulate human antigen-presenting cells (APCs) phenotype and to counteract their pro-invasive capacity. Interestingly, Ch/γ-PGA NPs had a prominent effect in inducing macrophages and dendritic cells immunostimulatory phenotype, thus favoring T cell proliferation and inhibiting colorectal cancer cell invasion. We propose that their combination with other immunomodulatory drugs or conventional anticancer therapies can improve patients' outcome.

1. Introduction

The immune system is a key regulator of tissue homeostasis but also of tumor development. At the tumor microenvironment,

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immune cells play a dual role with potential to either eliminate or promote cancer cell activities [1,2]. When these cells are chronically activated, they can support tumor cell proliferation and survival, culminating in the growth and development of neoplastic lesions. Several studies have shown that unresolved chronic inflammation at the tumor site recruits a diversity of cells with immunosuppressive activity [3], such as immature or dysfunctional dendritic cells (DCs), anti-inflammatory macrophages, myeloid-derived suppressor cells (MSDCs), regulatory T cells (Tregs) and T helper 2 (Th2) cells which favor immune escape and tumor progression [4,5]. From these cells, myeloid leukocytes have been recognized as the major driving force of tumor progression. Paradoxically, myeloid cells with antigen-presentation capabilities are also necessary for the initiation of tumor-specific T cell responses [5,6].

Macrophages and DCs work as sentinels in our body, and are required for a rapid response to foreign stimuli and to initiate a robust inflammatory reaction. Due to their phenotypic and functional plasticity, these cells are highly responsive to signals within the tumor microenvironment [7,8]. Tumor-associated macrophages (TAMs) play an important role in tumorigenesis [9,10] and accordingly, high levels of macrophage infiltration are frequently associated with poor cancer prognosis [11]. Although macrophage phenotype classification comprises a wide spectrum, it is usually accepted that they may differentiate into two opposite phenotypes: the classically activated M1 and the alternatively activated M2 macrophages [12,13]. M1-like macrophages are generally induced by interferon-gamma (IFN-γ), tumor-necrosis factor alpha (TNF-α), microbial products, as lipopolysaccharides (LPS) or by granulocyte-macrophage colony-stimulating factor (GM-CSF) [13]. They are associated with pro-inflammatory, microbicidal and tumoricidal activities, high antigen presentation abilities and high secretion of interleukin (IL)-12, IL-23 and IL-6, TNF-α, nitric oxide (NO), reactive oxygen intermediates (ROI) and low IL-10 levels. In contrast, M2-like macrophages may be induced by IL-4, IL-13, IL-10, M-CSF and glucocorticoid hormones. They generally present low levels of IL-12 and IL-6, high levels of IL-10, have the ability to scavenger and promote tissue repair, proteolysis, angiogenesis and tumor progression [14]. Within the tumor microenvironment, several phenotypes of myeloid cells can co-exist, but TAMs are usually phenotypically and functionally similar to M2-like macrophages [15–17]. Recently, our group demonstrated that IL-10-treated macrophages stimulate gastric and colorectal (CRC) cancer cell invasion, migration and proteolysis, through the phosphorylation of the cancer cell epidermal growth factor receptor (EGFR) and its downstream signaling partners, with matrix metalloproteinases (MMPs) having a central role in this process [18].

Similarly to macrophages, DCs constitute a specialized group of antigen-presenting cells (APCs) with high plasticity and their differentiation, maturation and activation are determined by microenvironmental cues [8]. DCs play a key role in cancer immunosurveillance, presentation of tumor antigens to naïve T cells, and consequently, priming and maintaining the antitumor immunity. Indeed, the enhanced infiltration of myeloid DCs in several human tumors has been associated with better prognosis [19]. However, at the tumor site, DCs maturation, activation and antigen-presenting capacity seem to be, at least partially, lost or inefficient, leading to tumor immune escape [20].

Considering the APCs plasticity and their essential role for an efficient antitumor immunity, several strategies have appeared to modulate the APCs phenotype at the tumor microenvironment, in order to potentiate tumor-specific immune responses.

Emerging nanotechnology for APCs-targeted therapies has been reported (reviewed in [21–24]) and these can be personalized to suppress, enhance, or subvert immune system recognition. Nanotherapies overcome some limitations of conventional therapies, namely the systemic distribution, high drug doses often required due to rapid clearance, poor drug selectivity, severe adverse effects, toxicity and the development of drug resistance. These therapies can also directly target immune and tumor cells, preventing immune tolerance and inducing antigen-specific immune responses. Different nanostrategies using lipids, poly-aminoacids, polysaccharides or iron oxide have been investigated for myeloid cell targeting *in vivo* [25–28]. Recently, our *in vitro* studies showed that IFN-γ incorporated into ultra-thin films, based on polyelectrolyte multi-layers (PEMs) of Chitosan (Ch) and Poly-γ(glutamic acid) (γ-PGA), modulated IL-10-stimulated macrophages towards a more pro-inflammatory phenotype and inhibited macrophage-mediated cancer cell invasion [29]. Despite the promising results, the delivery system above used, mounted as thin films over glass substrates could not be applied *in vivo*. This work was the premise to develop an injectable immunomodulatory system for *in vivo* applications based on Ch and γ-PGA biological properties.

Ch is a natural biodegradable polysaccharide and it is a cationic polymer, at pH below 6.5 [30]. Ch appeal is due to its biochemical activity, biocompatibility, nontoxicity, as well as its immunomodulatory properties [31]. γ-PGA is a hydrophilic, non-toxic poly-aminoacid, negatively charged at pH above 2.2 and its biodegradability into glutamate residues makes it appealing for biomedical applications [32,33]. Ch and γ-PGA can assemble into the form of nanoparticles (NPs) by co-acervation method [33]. We have recently reported that these NPs can be used as an anti-inflammatory drug delivery system, efficiently delivering the drug to target cells, as assessed by the inhibition of macrophage activation *in vitro* [34] and attenuation of the pro-inflammatory status of degenerated intervertebral discs [35]. However, the immunomodulatory potential of Ch/γ-PGA NPs by themselves on human cells, devoided of any pro- or anti-inflammatory molecule, was not yet explored.

Thus, the aims of the present work were to evaluate Ch/γ-PGA NPs ability to (1) modulate human macrophages and DCs towards an immunostimulatory profile; (2) promote activation of T cell responses by these APCs and (3) counteract APCs pro-invasive capacity. This report highlights the potential of using Ch/γ-PGA NPs as a future therapeutic strategy, based on its immunostimulatory properties and ability to target myeloid cells at the tumor microenvironment, favoring T cell immunity and hampering their cooperation in cancer cell-related activities, such as invasion.

2. Material and methods

2.1. Ch/γ-PGA NPs preparation

Ch/γ-PGA NPs were prepared by co-acervation method as previously described by our team [33]. Briefly, Ch (France-Chitine) was purified and characterized according to Antunes *et al.* [36]. Ch with the degree of acetylation (DA) of 10.4 ± 1.6%, determined by Fourier transform infrared spectrometry using KBr pellets (FTIR-KBr), and molecular weight (MW) of 324 ± 27 kDa, determined by size-exclusion chromatography, was used. γ-PGA (MW of 10–50 kDa; purity level of 99.5%) was produced from *Bacillus subtilis* cultures as described by Pereira *et al.* [33]. Ch/γ-PGA NPs were prepared at a molar ratio of 1:1.5 (mol Ch:mol γ-PGA). Solutions of Ch (0.2 mg/mL in 0.2 M AcOH) and γ-PGA (0.2 mg/mL in 0.05 M Tris-HCl with 0.15 M NaCl buffer solution) were combined by co-acervation method, in which a γ-PGA solution was dropped to a Ch solution, using a 1 mL syringe associated to a syringe pump (KD Scientific Inc.), at constant speed (3.6 μL/s), under high stirring and at room temperature (RT) (Fig. S1). All the solutions had the pH adjusted to 5.0. Ch/γ-PGA NPs were concentrated 10 times by centrifugation (13000 rpm, 30 min 4 °C) after initial preparation,

followed by resuspension in one tenth of the initial volume of buffer solution, at pH 5.0. For subsequent experiments, concentrated NPs were always used at final concentration of 1% (v/v) to minimize pH alterations and guarantee the maintenance of cell viability and metabolic activity. The endotoxin levels were measured in the NPs suspension by limulous amoebocyte lysate test (Lonza) and the levels were 0.041 EU/mL, which is negative according to the test used (only positive if $\geq$ 0.125 EU/mL).

2.2. Ch/γ-PGA NPs characterization

Ch/γ-PGA NPs were characterized in terms of their size, polydispersion index and zeta potential by dynamic light scattering (DLS) in a ZetaSizer Nano Zs (Malvern Instruments), equipped with a He-Ne laser (λ = 633 nm), as described elsewhere [34]. Each NPs batch produced was tested and 3 measurements per sample were acquired.

2.3. Ethics statement

Human samples obtained and procedures were performed in agreement with the principles of the Declaration of Helsinki. Monocytes were isolated from surplus buffy coats from healthy blood donors. These were kindly provided by the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ) from Porto, Portugal. This is covered by the ethical approval of the service, under which blood donors give informed written consent for the byproducts of their blood collections to be used for research purposes (Protocol reference 260/11).

2.4. Human monocyte isolation and macrophage/DC differentiation

Human monocytes were isolated from buffy coats from healthy blood donors, as previously optimized in our lab [18]. Briefly, peripheral blood mononuclear cells (PBMC) were collected from centrifuged buffy coats (30 min, 1200 g, RT, without brake) and incubated with RosetteSep human monocyte enrichment kit (StemCell Technologies), according to manufacturer's instructions. The mixture was diluted 1:1 with PBS supplemented with 2% FBS (heat inactivated fetal bovine serum, Biowest), layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged as before. The enriched monocyte layer was collected and washed with PBS. Following this procedure, over 80% of isolated cells were found to be CD14-positive. For monocyte-macrophage differentiation, 0.1×10^6 monocytes/3.8 cm^2 (24-wells plate) were cultured for 10 days in complete RPMI1640 medium, supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin, in the absence of M-CSF or other exogenous factors. For M1-like (LPS) and M2-like (IL-10) polarization, macrophages were incubated with 10 ng/mL LPS (Sigma-Aldrich) or IL-10 (ImmunotoTools), respectively, for an additional 72 h. Ch/γ-PGA NPs (0.7 mg/mL) were added to IL-10-stimulated macrophages at 1% (v/v) after 4 h of IL-10 stimulation, for 72 h.

For monocyte-DC differentiation, 2.0×10^6 monocytes/9.6 cm^2 (6-wells plate) were differentiated for 5 days in complete RPMI1640 medium, further supplemented with 50 ng/mL IL-4 and GM-CSF (Immunotools). Ch/γ-PGA NPs (0.7 mg/mL) were added to DCs, (corresponding to 1% v/v) for 48 h, and controls were left unstimulated. LPS (25 ng/mL) was used as positive maturation control.

2.5. Cell metabolic activity and viability

DC and macrophage cell cultures were observed daily under a light microscope (Olympus) from day 5 and 7, respectively, and images were acquired (Fig. S2). DCs and macrophages viability was measured using Annexin V Apoptosis Detection kit (BD Biosciences) that allows the identification of cell death either by necrosis and apoptosis, at days 7 and 13, respectively. Briefly, Annexin V-FITC and propidium iodide (PI) staining was performed for 15 min in a Binding buffer at 4 °C. For each sample, 1.0×10^4 cells were acquired using a FACS Canto Cytometer (BD Biosciences) with BD FACSDiva software. Results were analyzed using FlowJo software version 10 (TreeStar, Inc.). The metabolic activity of these cells was determined by resazurin reduction assay. Briefly, 48 h or 72 h after NPs addition, cells were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich) for 4 h at 37 °C and 5%CO_2. Fluorescence intensity was measured at 590 nm in a Synergy MX plate reader (BioTek), and values normalized to unstimulated cells.

2.6. Internalization of ftNPs by macrophages

Internalization of ftNPs was quantified by imaging flow cytometry (ImageStreamX®, Amnis, EDM Millipore) and confocal laser-scanning microscopy. For imaging flow cytometry analysis, macrophages were stimulated with IL-10 and incubated with ftNPs for 72 h. Afterwards, macrophages were harvested with accutase (eBioscience) at 37 °C during 30 min followed by gently scrapping. Cell suspension was washed once with PBS and fixed in paraformaldehyde (PFA) 1% during 15 min at RT. IL-10-stimulated macrophages without NPs were used as control. ftCh fluorescence was assessed by using a 488 nm laser for excitation and images were acquired in channel 2 (505–560 nm) from the CCD camera using a 40× objective (0.75 NA). For brightfield images, a brightfield LED lamp was used, and images were collected on channel 1, and at least 5×10^4 events were collected. Image analysis was performed using IDEAS® data analysis software (Amnis, EDM Millipore, version 6.2.64.0), following the Internalization wizard pipeline. For comparison purposes, the internalization score was calculated. This feature quantifies the ratio intensity of the ftCh fluorescence signal inside the cell to the intensity of the entire cell. To discriminate internalized *versus* membrane-associated NPs, we designed a mask for the whole cell, defined in the brightfield image (Channel 1), and a cytoplasmic mask (internal) performed by eroding the whole cell mask by 4 pixels. The mask used for the cytoplasm can be seen in Fig. 3a. To further determine the fate on NPs inside the cells, ftNPs internalization by macrophages was analyzed by confocal laser scanning microscopy (CLSM). ftNPs were added to macrophages cultures, as previously described [34]. After 72 h of incubation, cells were washed with PBS and fixed as above. Cells were washed with PBS, quenched with 50 mM NH_4Cl for 10 min and washed again. Cell membrane was permeabilized with 0.2% v/v Triton X-100 (Sigma-Aldrich) during 5 min and blocked with 5% (w/v) bovine serum albumin (BSA) for 45 min. Early endosomes were labeled with a specific anti-EEA1 antibody (clone 281.7, Santa Cruz) for 1 h, followed by goat-anti mouse AlexaFluor-647 antibody (Invitrogen) incubation, for 1 h in the dark. Cell nuclei were counterstained with DAPI. Coverslips were mounted with VectaShield and imaged by CLSM (Leica LSC SP2 AOBS, Leica Microsystems) and analyzed using ImageJ 1.43 software.

2.7. DC and macrophage profile evaluation

For cell surface receptor expression analysis, NPs were added to DC and macrophage cell cultures for 48 or 72 h, respectively. Afterwards, DCs in suspension were harvested and macrophages were incubated with accutase (eBioscience) at 37 °C during 30 min and harvested by gently scrapping. Cells were washed and resuspended in FACS buffer (PBS, 2% FBS (Biowest), 0.01% sodium azide) containing appropriate conjugated antibodies, and stained in the dark for 45 min at 4 °C. DCs were immunostained with the following

antibodies: anti-human CD1a-APC (clone HI149), CD11c-APC (clone BU15), HLA-DR-PE (clone MEM-12), CD86-FITC (clone BU63), CD40-PE (clone HI40a) (Immunotools) and CD83-FITC (clone HB15e) (AbDSerotec). Macrophages were immunostained with the following antibodies: anti-human CD14-APC (clone MEM-18), HLA-DR-FITC (clone MEM-12) (Immunotools) and CD163-PE (clone GHI/61) (R&D Systems). To define background staining isotype-matched antibodies were used as negative controls. After additional washing steps, cells were acquired on a FACS Canto Flow Cytometer and analyzed as above. Median fluorescence intensity was calculated by subtracting the respective isotype control intensity.

2.8. DC and macrophage profile by ELISA

DCs and macrophages supernatants were assayed after 48 h or 72 h of NPs stimulation, respectively. IL-6, IL-12p40, IL-10 and TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA) TMB Development kit (Peprotech), according to manufacturer's instructions. IL12-p70, IL-23 and IFN-γ levels in culture media of DC, macrophage cultures and in coculture with T cells were measured by Legend Max ELISA (Biolegend), according to manufacturer's instructions.

2.9. Analysis of T cell proliferation

Monocytes were plated at 1×10^5 and 2×10^5 cells per well for macrophage and DC differentiation, respectively, and then stimulated as above described. Enriched lymphocyte populations were obtained from buffy coats of different donors by centrifugating over Lymphoprep (800 g, 30 min without brake). Following this procedure, 60% of isolated cells were found to be $CD3^+$. To remove monocyte contaminants, the PBMC fraction was washed with PBS and plated for 2 h at 37 °C, allowing monocytes to adhere. The non-adherent lymphocyte fraction was collected and 1×10^7 lymphocytes/mL were labeled with 1 mM CFSE (Invitrogen), in PBS (37 °C, 15 min), followed by two washes (5 min, 2500 rpm) in PBS with 20% FBS. An enriched T cell suspension was then resuspended in complete RPMI1640 medium and 0.8×10^6 and 2×10^6 cells were added to macrophages (8:1 ratio) and to DCs (10:1 ratio), respectively [37,38], or cultured alone with DC's (IL-4 and GM-CSF) or macrophage (LPS or IL-10) differentiating cytokines, and NPs. Phytohemagglutinin (PHA, Sigma-Aldrich) stimulation was used as positive proliferation control. After 7 days, cells were harvested and surface labeled with specific anti-human antibodies for CD3-APC (clone UCHT-1), CD4-PEDy647 (clone EDU-2) and CD8-PE (clone UCHT-4) (Immunotools), and analyzed by flow cytometry, as above described. T cell division was determined by the extent of CFSE halving, on $CD3^+$ $CD4^+$ cells and on $CD3^+$ $CD8^+$ cells.

2.10. Matrigel invasion assay

RKO cells (5×10^4) were seeded on the upper compartment of Matrigel-coated inserts with 8-μm pore size (BD Biosciences), while non-activated and activated DCs, or non-polarized and polarized macrophages (1×10^5) in contact with NPs were added on the lower compartment, as previously described [18]. After 24 h, invasive cells were counterstained with DAPI and visualized using a fluorescence light microscope (Leica). The total number of invasive RKO cells was counted using a 20× objective, considering only the nuclei that completely passed through the pores.

2.11. Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v5 (GraphPad-v5). Due to the limited number of donors included in test groups ($n < 7$), a non-Gaussian distribution of the data was considered for the statistical analysis. Therefore, the non-parametric Friedman test was used for paired comparisons between groups. Statistical significance was achieved when $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Kruskal-Wallis test was used for non-pared comparisons between groups. Statistical significance was achieved when $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Characterization of Ch/γ-PGA NPs

In the current study, we investigated the potential of Ch/γ-PGA NPs to modulate human antigen-presenting cells, and consequently to counteract cancer-cell invasion. Thus, Ch/γ-PGA NPs were prepared by dropping a γ-PGA solution, at constant rate and under stirring at pH 5, to a Ch solution, as previously described [33,34] (Fig. S1a). The molar ratio, polymer concentration and pH of interaction were first optimized to obtain a low polydisperse solution with nano-sized particles of Ch and γ-PGA [33]. Particles size, polydispersion index and zeta potential were evaluated by Dynamic light scattering (DLS) and are summarized in Table 1. Our results indicated that Ch/γ-PGA NPs presented a consistent size (183.0 ± 2.8 nm) with a reduced polydispersion index (0.20 ± 0.01). The zeta potential of Ch/γ-PGA NPs was 18.7 ± 0.8 mV.

3.2. Ch/γ-PGA NPs do not affect APCs viability nor metabolic activity

APCs, namely macrophages and DCs, are crucial for the generation and maintenance of an effective antitumor immune response. Since these cells have a protumoral role in certain types of cancer, the development of APCs-targeted therapies is essential for an efficient treatment [6]. To evaluate the safety of Ch/γ-PGA NPs administration, human DC and macrophage cultures were observed for apoptosis-related morphological features, and their viability and mitochondrial metabolic activity were assessed. Morphologically, no apoptotic signs, such as cell shrinkage, pyknosis or loss of membrane integrity, were observed in Ch/γ-PGA NPs-stimulated DCs or IL-10-stimulated macrophages treated with NPs, excluding possible toxic effects on cell cultures. DCs stimulated with Ch/γ-PGA NPs were larger, similar to LPS-activated DCs, with cytoplasmic projections and abundant cytoplasm, while unstimulated DCs had small dendrites and no aggregates. Ch/γ-PGA NPs induced a more elongated macrophage phenotype with reduced podosome-like structures, both features of LPS-treated macrophages [29] (Fig. S2). Additionally, the distinct treatments did not affect DC and macrophage viability, as demonstrated by the Annexin/PI staining (Fig. 1a), nor their metabolic activity, as demonstrated by the Rezasurin assay (Fig. 1b). In summary, no significant

Table 1
Characterization of Ch/γ-PGA nanoparticles. Size (nm), polydispersity index (PdI) and zeta potential (mV) of Ch/γ-PGA NPs determined by dynamic light scattering (DLS) shortly after NPs preparation. Results are presented as mean ± SEM (n = 3).

	Particle size (nm)	Polydispersity index	Zeta potential (mV)
Ch/γ-PGA NPs	183.0 ± 2.8	0.2 ± 0.01	18.7 ± 0.8

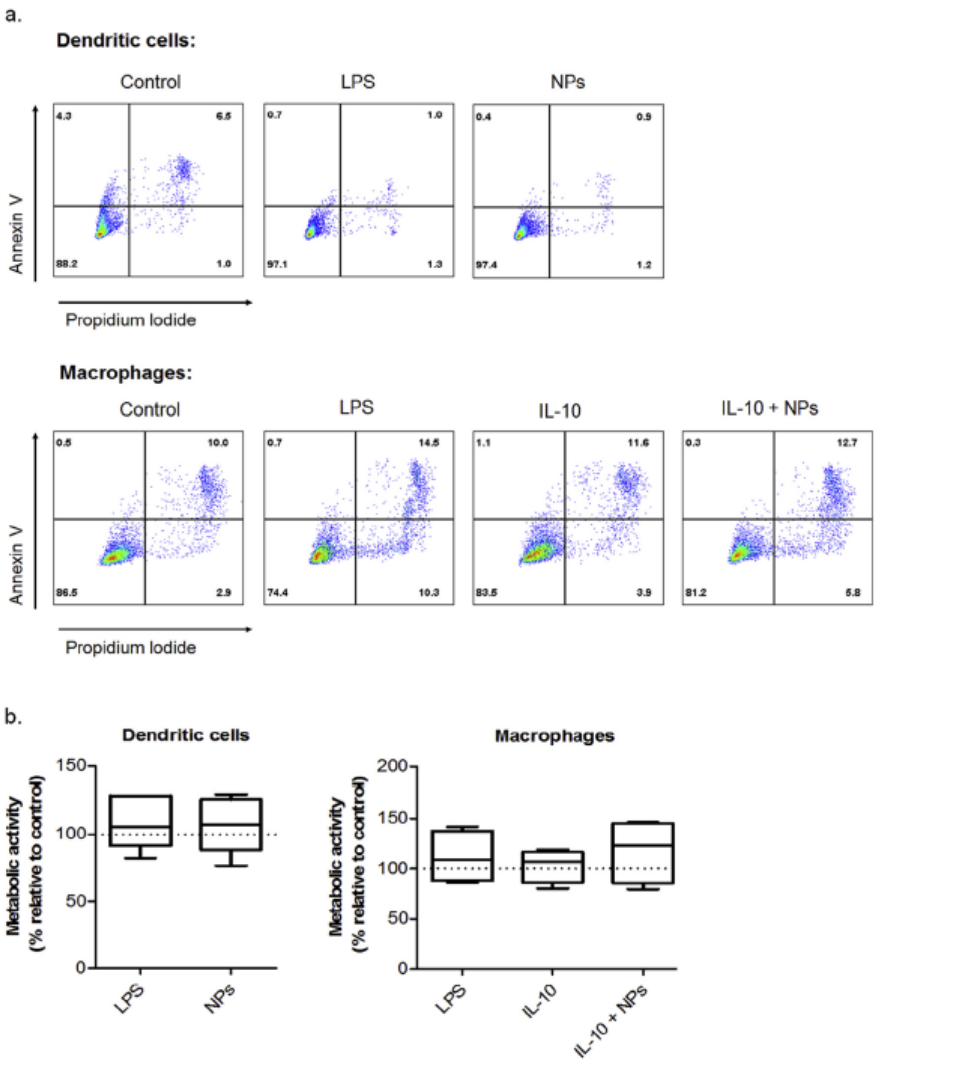

Fig. 1. Ch/γ-PGA NPs do not affect human DC and macrophage viability nor metabolic activity. Human DCs were differentiated in the presence of GM-CSF and IL-4 during 5 days. Afterwards, DCs were cultured with Ch/γ-PGA NPs for an additional 48 h. Macrophages were differentiated in culture medium for 10 days and were incubated with LPs or IL-10 for 72 h, to obtain M1- or M2-like macrophages, respectively. IL-10-stimulated macrophages were additionally stimulated with Ch/γ-PGA NPs for 72 h. a. DC (upper panel) and macrophage (lower panel) viability was determined by staining for cell surface Annexin V and Propidium Iodide (PI) followed by flow cytometry analysis. Annexin V vs PI plots from gated single cells show the populations corresponding to viable and non-apoptotic (Annexin V$^-$ PI$^-$), necrotic cells (Annexin V$^-$ PI$^+$), early (Annexin V$^+$ PI$^-$), and late (Annexin V$^+$ PI$^+$) apoptotic cells. Numbers in gates indicate the percentage of these populations. b. Metabolic activity was evaluated by Rezasurin assay and compared with control cells (unstimulated DCs or macrophages). Median is represented by the horizontal line inside the box plots. Data is representative of at least 4 different blood donors. All comparisons were performed using the Friedman test followed by Dunn's multiple comparison test relative to unstimulated DCs or macrophages.

alterations in cell morphology, viability and metabolic activity were detected, indicating that treatment with 0.7 mg/mL of Ch/γ-PGA NPs at pH 5 is biologically safe. These results further support the use of this Ch/γ-PGA formulation for *in vivo* administration, since the addition of the NPs at pH 5 did not significantly acidified the culture medium, nor created a toxic or inhibitory cell culture environment.

3.3. Ch/γ-PGA NPs induce DC maturation/activation

DCs activation is an essential requirement for an effective anti-tumor response [39]. However, at the tumor site, DCs differentiation is often hindered, resulting in the recruitment and accumulation of functionally deficient and immature DCs [20]. To determine whether Ch/γ-PGA NPs could promote DC maturation/

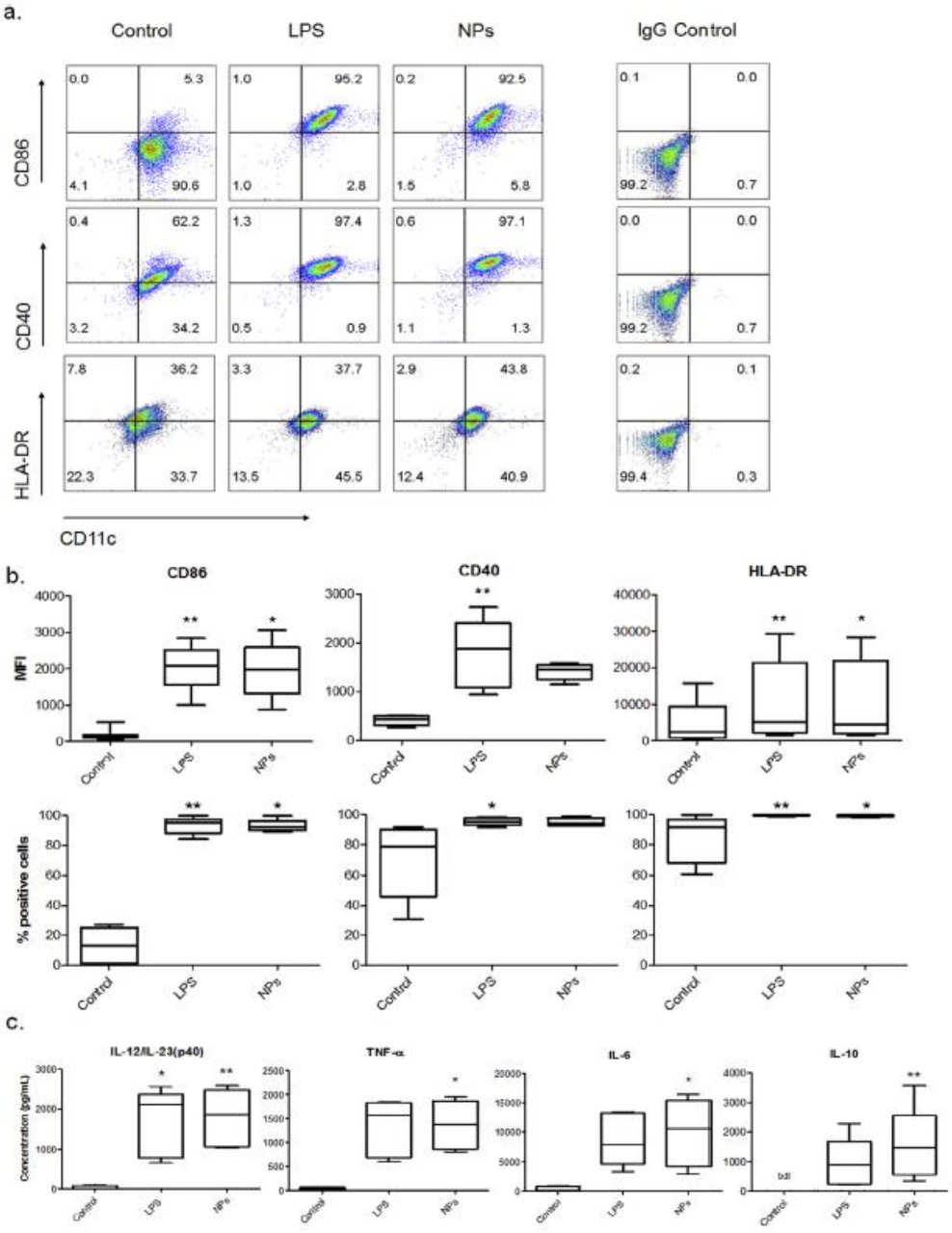

Fig. 2. Ch/γ-PGA NPs induce DC maturation/activation. Differentiated DCs were stimulated with LPS, as positive control, or with Ch/γ-PGA NPs for 48 h. Cells were surface stained for the lineage marker CD11c and the activation/maturation markers CD86, CD40 and HLA-DR and analyzed by flow cytometry. a. The pseudocolor plot of a representative phenotypic profile shows the electronic gate used to identify $CD11c^+$ $CD86^+$ cells, $CD11c^+$ $CD40^+$ and $CD11c^+$ $HLA\text{-}DR^+$ cells gated on single cells. Numbers indicate the percentage of the above cited populations. b. Top panel: The median fluorescence intensity (MFI) of CD86, CD40 and HLA-DR was determined by subtracting the fluorescence intensity of the respective isotype control; Bottom panel: percentage of positive cells was determined. Data of at least 6 donors is represented in box plots, where the horizontal line indicates the median. c. DC conditioned media were recovered and levels of the pro-inflammatory cytokines IL-12/IL-23(p40), TNF-α and IL-6, and of the anti-inflammatory cytokine IL-10 were determined by ELISA. Data of at least 5 donors is represented in box plots, where the horizontal line indicates the median. Bdl means below detection level of ELISA kit. In all cases, comparisons were performed using the Friedman test followed by Dunn's multiple comparison test relative to unstimulated DCs ($^*p < 0.05$; $^{**}p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation, we studied their ability to modulate the surface expression of the maturation and activation molecules CD86, CD40, the major histocompatibility complex-II molecule HLA-DR and CD83, by flow cytometry. Importantly, for all the conditions tested, the percentage of cells expressing the lineage marker CD11c was maintained, indicating no dedifferentiation (Fig. 2a). The Toll-like receptor (TLR) agonist LPS served as positive control to promote DC maturation and, as expected, it strongly enhanced both the median intensity fluorescence (MFI) and the percentage of cells that expressed CD86, CD40 and HLA-DR. Interestingly, Ch/γ-PGA NPs also increased DCs surface expression of these molecules, to levels comparable to LPS (Fig. 2b). Further, the expression of the maturation marker CD83 revealed a significant increase of both MFI and percentage of positive cells after Ch/γ-PGA NPs treatment (Fig. S3). DC maturation/activation was further confirmed by cytokine secretion profile. Ch/γ-PGA NPs stimulated the production of pro-inflammatory cytokines IL-12/IL-23(p40), TNF-α and IL-6 relatively to unstimulated DCs, similarly to LPS (Fig. 2c). By analyzing separately IL-12p70 and IL-23, it was possible to observe that IL-12p70 rather than IL-23 was the main contributor for the IL-12/IL-23(p40) increase (Fig. S4). Curiously, IL-10 levels also increased after Ch/γ-PGA treatment. Altogether, these results suggest that Ch/γ-PGA NPs induce a DC immunogenic profile.

3.4. Ch/γ-PGA NPs prevent IL-10-induced macrophage polarization

Macrophages represent the major stromal inflammatory component of many tumors and, these cells frequently have associated protumoral functions and are functionally similar to M2-like macrophages [40]. Therapies favoring a pro-inflammatory/immunostimulatory profile (M1-like) might improve conventional anticancer therapies.

At first, the ability of IL-10-stimulated macrophages to internalize the Ch/γ-PGA NPs was determined. Using previously labeled Ch with FITC (ft), the Ch/γ-PGA NPs internalization by IL-10-stimulated macrophages was evaluated after 72 h by Imaging Flow Cytometry and by confocal microscopy. Regarding the first analysis, single cells were selected, and by applying an internalization mask on the brightfield image (Fig. 3a), the internalization score (ratio between FITC fluorescence intensity inside the cell and FITC fluorescence intensity of the entire cell) was determined. Representative dot plot and histogram profiles for the ft(Ch/γ-PGA NPs) internalization by human IL-10-stimulated macrophages are represented in Fig. 3b, with about 64.7 ± 0.7% of total macrophages with internalized ft(Ch/γ-PGA NPs) (ftNPs$^+$ cells). NPs cellular localization (cytoplasmic or membranar) was also determined within ftNPs$^+$ cells, revealing that 98.2 ± 0.2% of macrophages had higher fluorescence in the cytoplasm, while 1.8 ± 0.2% presented higher fluorescence intensity at the cell membrane, indicating a high efficiency of internalization. After confirming that IL-10-stimulated macrophages had the ability to internalize NPs, the Ch/γ-PGA NPs subcellular localization was analyzed by confocal microscopy. Macrophages were stained with an early endosome marker (EEA-1), while nuclei were counterstained with DAPI. Orthogonal projections in XZ and YZ were used to evaluate NPs internalization. We observed that, although some of ft(Ch/γ-PGA NPs) aggregates were located outside the cell membrane, the majority of the ft(Ch/γ-PGA NPs) were internalized and localized in the cytoplasm, around the nucleus (Fig. 3c).

To determine whether Ch/γ-PGA NPs have the ability to inhibit M2 polarization, Ch/γ-PGA NPs were added to IL-10-stimulated macrophages and the surface expression of HLA-DR and CD163, M1- and M2-like markers respectively, was evaluated by flow cytometry (Fig. 4a). LPS or IL-10-treated macrophages were also analyzed for control purposes. Ch/γ-PGA NPs treatment seemed to slightly increase the MFI for the lineage marker CD14, although not reaching statistical significance (Fig. 4b). As expected, LPS stimulation decreased the number of CD163$^+$ cells, while IL-10 stimulation had the opposite effect. Notably, IL-10-stimulated macrophages treated with Ch/γ-PGA NPs showed a reduction of both CD163 MFI and the percentage of CD163$^+$ cells (Fig. 4b), suggesting that Ch/γ-PGA NPs by themselves prevent macrophage M2-like polarization by IL-10. Surprisingly, the HLA-DR expression was not affected by LPS or Ch/γ-PGA NPs treatment. In order to further characterize the macrophage phenotype induced by NPs stimulation, we profiled the cytokine production ability of these cells. In agreement with the results for surface markers expression, LPS stimulation, and not IL-10, increased the production of IL-12/IL-23(p40), TNF-α and IL-6. Interestingly, IL-10-stimulated macrophages treated with Ch/γ-PGA NPs induced a significant increase in IL-12/IL-23(p40) and TNF-α, while IL-10 were not altered (Fig. 4c). It was not possible to discriminate between IL-12p70 and IL-23 since the levels were below the detection limit. Overall, the decrease in CD163 levels on IL-10-stimulated macrophages treated with Ch/γ-PGA NPs together with the increase of pro-inflammatory cytokines suggests that NPs inhibit M2 polarization, inducing a pro-inflammatory profile even in the presence of an IL-10 environment.

3.5. Ch/γ-PGA NPs induce T cell proliferation

Antitumor immune responses have been mainly attributed to CD8T cells activity. However, CD4T cells also play a central role in this process. The ability of activated DCs and macrophages to induce T cell activation influences the adaptive immune response at the tumor microenvironment [41]. To understand the influence of Ch/γ-PGA NPs on DC and macrophage function, DC- and macrophage-induced T cell proliferation was evaluated. Differentiated DCs and macrophages stimulated with Ch/γ-PGA NPs were cultured with CFSE-labeled lymphocytes isolated from a different blood donor, setting up a mixed lymphocyte reaction. Seven days after co-culture, lymphocytes were recovered and stained with antibodies specific for CD3, CD4 and CD8 surface markers (Fig. S5a). As experimental controls, CFSE-lymphocytes were cultured alone, with GM-CSF and IL-4, LPS or IL-10, whereas PHA stimulation was the positive control. As expected, CFSE-lymphocytes alone or with GM-CSF and IL-4, LPS or IL-10 did not proliferate, while PHA induced T cell proliferation in a dose-dependent manner (Fig. S5b,c). In agreement with previous reports [42], our results evidenced that monocyte-derived DCs (CD11c$^+$ cells) had the capacity of inducing T cell proliferation (Fig. 5 a,b). LPS-stimulated DCs significantly increased the percentage of CD4-dividing cells when compared to unstimulated DCs. Although LPS-stimulated DCs also increase CD8$^+$ T cell proliferation, no statistical significance was reached. Interestingly, similar results were obtained with Ch/γ-PGA NPs (Fig. 5 a,b). To exclude the possibility that Ch/γ-PGA NPs could induce T cell proliferation independently of DCs, T-lymphocytes were stimulated with Ch/γ-PGA NPs alone (Fig. S5b,c) or with the most abundant DCs-produced cytokines, specifically, IL-6, IL-12, TNF-α, in similar concentrations as the ones detected in our system (Fig. S5d). The results confirmed that the effect of Ch/γ-PGA NPs on T cell proliferation was DCs dependent. Nevertheless, we cannot exclude the presence of other factors in the conditioned medium, produced after stimulation, which might contribute to T cell proliferation. Additionally, DCs stimulated with NPs in contact with T cells significantly exhibited higher levels of IFN-γ in comparison to NPs-stimulated DCs or NPs-stimulated T cells (Fig. 5c)

To evaluate the capacity of macrophages to drive T cell responses, macrophages stimulated with LPS, IL-10 or with IL-10 together with Ch/γ-PGA NPs were co-cultured with lymphocytes during 7 days, and T cell proliferation was evaluated. Similarly to

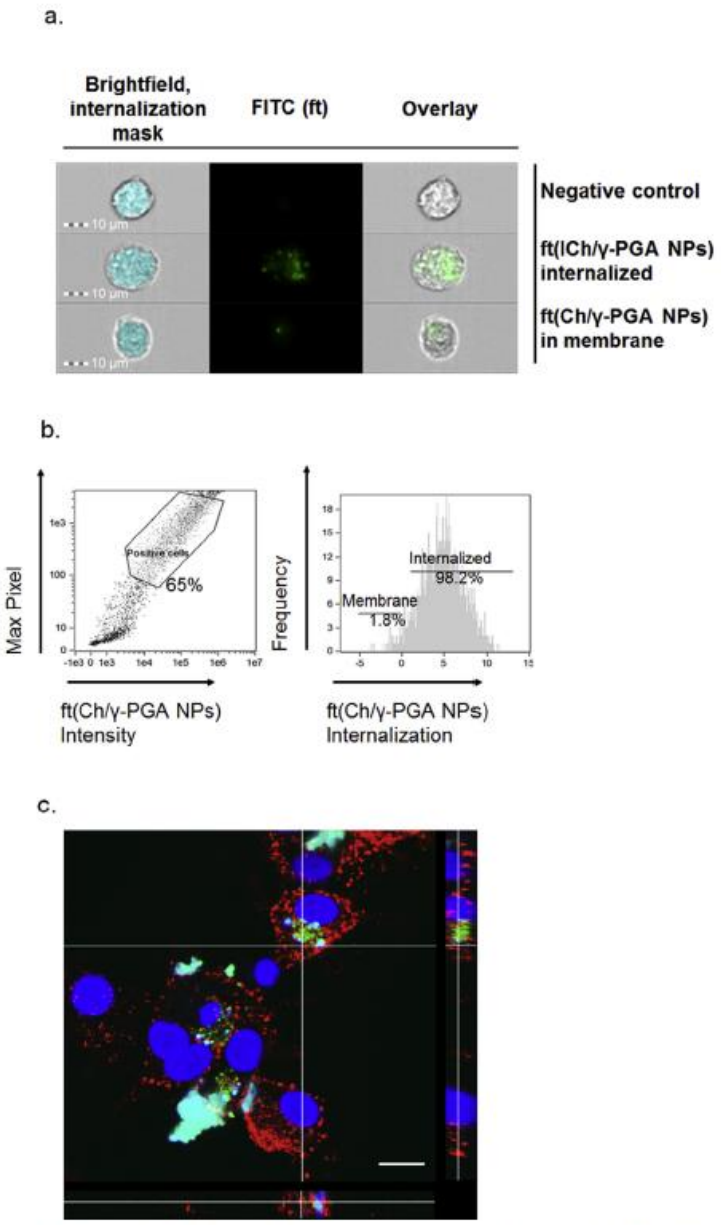

Fig. 3. ft(Ch/γ-PGA NPs) are internalized by macrophages. Human macrophages were stimulated with IL-10 alone or with Ch/γ-PGA NPs for 72 h. Cells were recovered and fixed for Imaging Flow Cytometry and confocal microscopy. a. The percentage of NPs internalization was determined after application of an internalization mask on the positive cell population of positive cells for FITC(ft) ft(Ch/γ-PGA NPs). Each cell is represented by a row of three images acquired simultaneously in flow, from left to right: brightfield with cytoplasm mask represented in blue, FITC fluorescence (green) for the ft(Ch/γ-PGA NPs), merged image (scale bars, 10 μm). b. Representative dot plot and histogram profile for the ft(Ch/γ-PGA NPs) internalization by macrophages (lower panel) by Imaging Flow Cytometry. c. IL-10-stimulated-macrophages treated with ft(Ch/γPGA -NPs) were stained with a specific anti-Eea1-Alexa647 antibody (in red), nuclei were counterstained with DAPI (in blue) and FITC stains the Ch from the ft(Ch/γ-PGA NPs) (in green). Orthogonal projections were created from z-stacks of single 16 images acquired at intervals of 1 μm. Scale bar represents 100 μm in the macrophage image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DCs, unstimulated macrophages induced T cell division (Fig. 5b). Notably, LPS-activated macrophages did not enhance $CD4^+$ T cell proliferation but induced significantly higher $CD8^+$ T cell proliferation in comparison to unstimulated or IL-10-treated macrophages. Interestingly, when IL10-stimulated macrophages were incubated with Ch/γ-PGA NPs, $CD8^+$ T cell proliferation was significantly

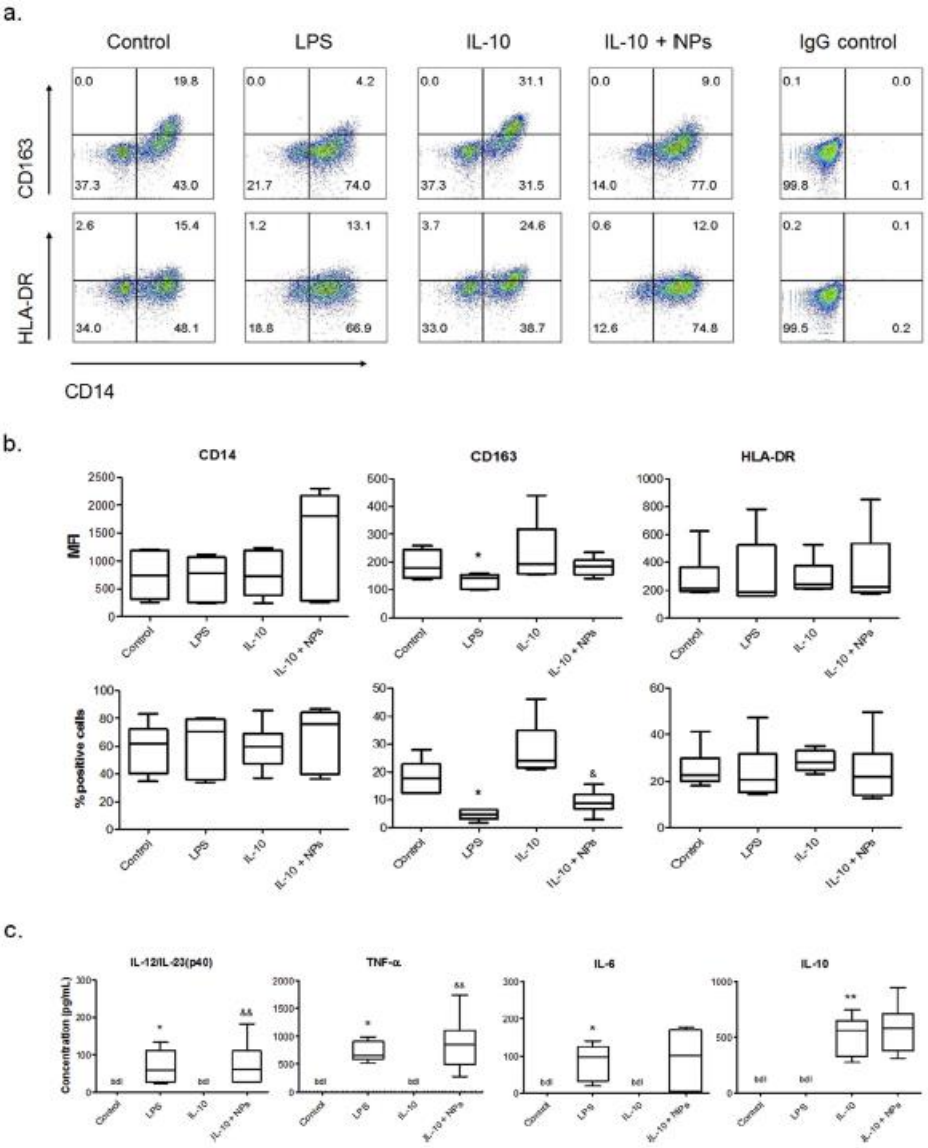

Fig. 4. Ch/γ-PGA NPs prevent an anti-inflammatory macrophage profile. Macrophages were stimulated with LPS or IL-10 during 72 h in order to polarize towards M1- or M2-like macrophage profile, respectively. Simultaneously, IL-10-stimulated macrophages were cultured with Ch/γ-PGA NPs for 72 h. Then, cells were surface stained for the monocyte/macrophage lineage marker (CD14), the M1 marker (HLA-DR), and the M2 marker (CD163), and were analyzed by flow cytometry. a. The pseudocolor plot of a representative phenotypic profile show the electronic gate used to identify $CD14^+$ $CD163^+$ cells and $CD14^+$ $HLA\text{-}DR^+$ cells gated on single cells. Numbers indicate the percentage of the above cited populations. b. Top panel: The MFI of CD14, CD163 and HLA-DR was determined by subtraction of the fluorescence intensity of the respective isotype control; Bottom panel: percentage of positive cells was determined. Data is representative of at least 6 donors. c. Macrophage conditioned media were recovered and levels of the pro-inflammatory cytokines IL-12/IL-23(p40), TNF-α and IL-6, and of the anti-inflammatory cytokine IL-10 were determined by ELISA (n = 6). Comparisons were performed using the Friedman test followed by Dunn's multiple comparison test ($^*p < 0.05$; $^{**}p < 0.01$ relative to unstimulated macrophages- control; $^{\&}p < 0,05$; $^{\&\&}p < 0.01$ relative to IL-10-stimulated macrophages). Median is represented by the horizontal line inside the box plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased. Moreover, IL-10-stimulated macrophages treated with NPs and in contact with T cells significantly produced higher levels of IFN-γ in comparison to NPs-stimulated IL-10-macrophages or NPs-stimulated T cells (Fig. 5c). Altogether, these results suggest that Ch/γ-PGA NPs could be used to promote T cell proliferation and activation, in response to APCs.

3.6. Ch/γ-PGA NPs inhibit macrophage and DC ability to promote colon cancer cell invasion

M2 macrophages have been reported as efficient stimulators of cancer cell invasion [43]. To evaluate the efficacy of Ch/γ-PGA NPs to counteract this stimulatory effect, Matrigel invasion assays con-

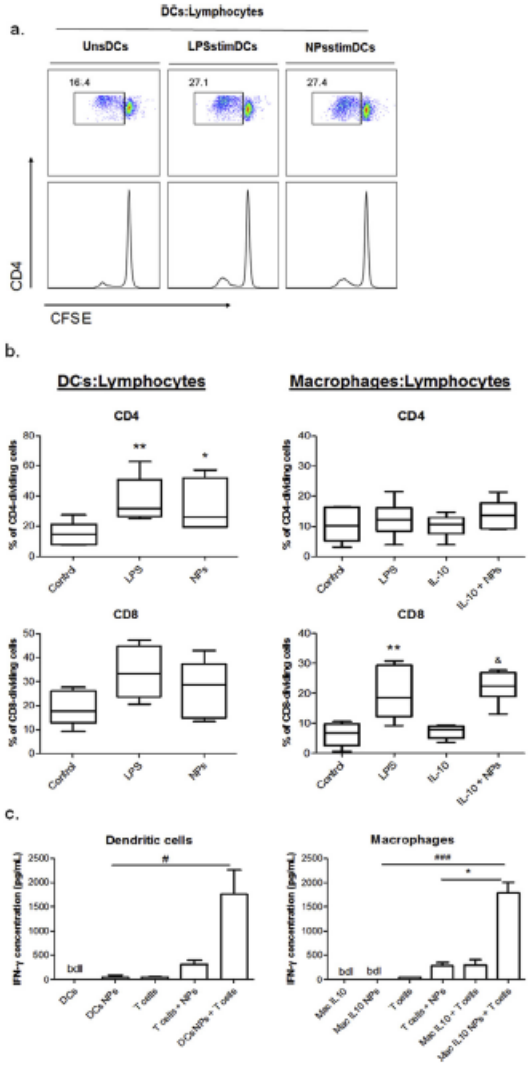

Fig. 5. DCs and macrophages stimulated with Ch/γ-PGA NPs are able to induce specific T cell proliferation. Lymphocytes isolated from a different blood donor labeled with allogeneic carboxyfluorescein succinimidyl ester (CFSE) were added to DCs stimulated with LPS or NPs at a ratio of 1:10 (DCs:lymphocytes) and to macrophage stimulated with LPS, IL-10 or IL-10 together with Ch/γ-PGA NPs (IL-10$^+$ NPs) at a ratio of 1:8 (macrophages:lymphocytes). Lymphocytes cultured with DCs differentiating cytokines (GM-CSF and IL-4), PHA, LPS and IL-10 were used as experimental controls. After 7 days of co-culture, T cells were surface stained for CD3, CD4 and CD8 and analyzed by flow cytometry. a. Representative plots of CD4 vs CFSE of lymphocytes co-cultured with unstimulated DCs, with LPS- or Ch/γ-PGA NPs-stimulated DCs. b. and c. The percentage of proliferating CD4 and CD8 cells, measured at day 7 of co-culture with DCs and macrophages, were expressed as % of dividing cells in response to the antigenic stimulus. Data is representative of at least 6 different blood donors and median is represented by the horizontal line inside the box plots. Comparisons were performed using the Friedman test followed by Dunn's multiple comparisons test ($^*p < 0.05$; $^{**}p < 0.01$ relative to control for DCs:lymphocytes co-culture; $^{**}p < 0.01$ relative to control and $^{\&}p < 0.05$ relative to IL-10-stimulated macrophages for macrophages:lymphocytes co-culture). c. DCs, macrophages and co-cultures with T cells conditioned media were recovered and levels of the IFN-γ was determined by ELISA ($n \geq 3$). Friedman test was used for paired comparisons between groups, followed by Dunn's multiple comparison test. Statistical significance was achieved when $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with single cultures, and $^*p < 0.05$ compared with T cells cultured with NPs only.

fronting human colon RKO cancer cells with IL-10-stimulated macrophages, treated or not with Ch/γ-PGA NPs, were performed (Fig. 6a). As expected, M2-like macrophages led to an increase of cancer cell invasion. Interestingly, Ch/γ-PGA NPs significantly decrease this invasion-promoting ability of IL-10-stimulated macrophages (0.51 ± 0.09) (Fig. 6b). These results indicate that Ch/γ-PGA NPs were sufficient to modulate IL-10-treated macrophages, and significantly decreased macrophage-mediated RKO cell invasion.

The role of DCs on cancer cell invasion remains unknown. To unveil their role, Matrigel invasion assays confronting human colon RKO cancer cells with unstimulated DCs were performed. Our results evidenced that DCs are less efficient than macrophages in stimulating RKO cancer cell invasion, with 70 ± 9 and 156 ± 44 invasive cells, respectively (data not shown). Furthermore, immature DCs were able to induce higher cell invasion than LPS-activated DCs (0.42 ± 0.10) (Fig. 6c). Similarly, DCs treated with Ch/γ-PGA NPs decreased the invasion ability of RKO cells (0.53 ± 0.05). Altogether, these results suggest that Ch/γ-PGA NPs can impair macrophage and DC-mediated cancer cell invasion.

4. Discussion

The development of new and improved antitumor therapies requires a comprehensive knowledge of innate immunity and how its activation translates into effective adaptive responses able to counteract the immunosuppressive tumor microenvironment. The demonstration of the key role of APCs in the induction of tolerance to tumor antigens prompts the search for new strategies to modulate the inflammatory status of these cells and, consequently influence the functional outcome of tumor antigen-specific T cells. The inclusion of adjuvants and immunomodulatory agents in therapeutic formulations can enhance the adaptive response, leading to the desired cellular phenotype [25,26,28]. Chitosan [31,44,45] and γ-PGA [46,47] are attractive adjuvants and their biodegradability, biocompatibility and nontoxicity provide a huge potential for novel therapeutic approaches. We have recently reported a strategy whereby incorporation of IFN-γ within Ch/γ-PGA self-assembled PEMs modulated human macrophages phenotype [29]. To translate this knowledge into an injectable therapy feasible *in vivo*, we produced a formulation of Ch/γ-PGA into NPs and tested their immunomodulatory activity. The selection of Ch/γ-PGA NPs was based on their successful application in the delivery of bioactive molecules. In particular, Ch/γ-PGA NPs were already described in the delivery of active substances such as SDF-1, Diclofenac and insulin [34,35,48]. Here, we explored the immunomodulatory potential of these NPs, investigating their ability to re-educate human DCs and macrophages, both crucial agents of innate immunity, to an antitumor phenotype and analyzed, for the first time, the ability of these Ch/γ-PGA NPs to counteract human macrophage and DC-mediated colorectal cancer cell invasion.

Aiming at evaluating Ch/γ-PGA NPs successful modulation of APCs phenotype *in vitro*, we first evaluated the cytotoxicity of these NPs and their impact on human macrophage and DC cultures. Our results indicated that Ch/γ-PGA NPs, at concentrations equal or below 0.7 mg/mL, did not affect the viability nor the metabolic activity of APCs. DCs activation and maturation was confirmed by surface markers expression. Notably, Ch/γ-PGA NPs enhanced CD86, HLA-DR, CD83 and CD40 expression and induced high levels of pro-inflammatory cytokines IL-6, IL-12/IL-23(p40) and TNF-α. Interestingly, these NPs also enhanced IL-10 levels, which can be a result of a regulatory feedback mechanism to limit DCs activation [49]. Functionally, DCs treated with NPs were efficient in activating allogeneic T cells, significantly enhancing CD4T cell response.

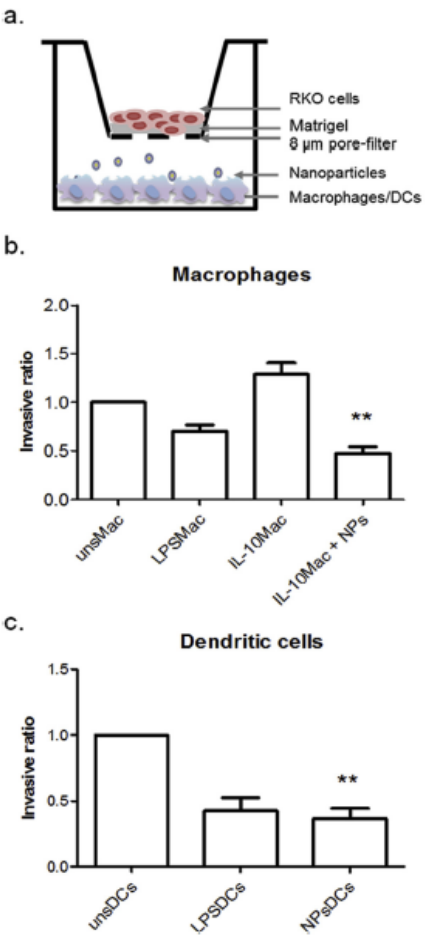

Fig. 6. Ch/γ-PGA NPs inhibit macrophage and DC ability to promote colon cancer cell invasion. a. Schematic representation of the Transwell invasion assay. Macrophages (b.) were treated with LPS, or IL-10, in the presence or not of NPs, and DCs (c.) were treated with LPS or NPs, and seeded in a well of a 24-well invasion plate. RKO colon cancer cells were seeded inside transwell inserts (with 8 μm-pore size membranes) coated with Matrigel, which were then placed above DC and macrophage cultures. After 24 h, insert membranes were removed and the number of invading cells were counterstained with DAPI, for counting under the microscope. Data show the Median ± SEM and it is representative of at least 3 donors. Comparisons were performed using the Friedman test followed by Dunn's multiple comparisons test ($^{**}p < 0.01$). The results were normalized to IL-10-stimulated macrophages or to unstimulated DCs.

As CD40 seems to be up-regulated together with higher levels of IL-12 produced by NPs-stimulated DCs, it is probable that DCs drive towards a T helper type 1 response [50]. These results demonstrate that Ch/γ-PGA NPs are able to revert the immature phenotype of DCs, often present at the tumor microenvironment, activating them into an immunostimulatory state.

Several strategies to target DCs have been explored, making use of compounds with intrinsic immunostimulatory properties, as TLR ligands (LPS, poly(I:C), imidazoquinolines) or with adjuvant properties that can comprise different functions and activities, including drug delivery, targeting or immunomodulatory properties [51,52]. Regarding immunoadjuvant compounds, γ-PGA NPs were tested on mouse spleen and bone marrow-derived DCs, and efficiently up-regulated the expression of co-stimulatory molecules and pro-inflammatory cytokines. This maturation was achieved through nuclear factor (NF-κβ) and mitogen-activated protein kinases (MAPK) signaling pathways activation. The ability to these NPs to induce a long-term cellular and humoral immunity was only achieved when combined with ovalbumin, demonstrating its efficiency as an antigen carrier to DCs [53,54]. Furthermore, our group demonstrated that Ch films induce a pro-inflammatory DC profile [42]. Additionally, tumor lysate-pulsed DCs previously cultured on Ch substrate increased DC activation and antitumor immunity, compared to tissue culture polystyrene (TCPS) [45]. More recently, Carroll *et al.* demonstrated that Ch promotes DC maturation and Th1 response in a type I IFN receptor-dependent manner [31]. However, their Ch-induced cell maturation did not coincide with IL-12p40 and IL-6 secretion, which suggests that other receptors and signaling partners might be involved in Ch recognition. These authors excluded the involvement of TLR4, but other reports pointed that Ch may induce DC maturation through a TLR4-dependent mechanism [55] or through inflammasome activation [56]. Ch origin, its degree of acetylation (DA) and formulation also affect physicochemical and immunological characteristics and, as a consequence, host immune responses.

Similarly, the effect of Ch/γ-PGA NPs on macrophage phenotype was studied by analyzing several phenotypic and functional aspects described for M2-like macrophages. Here, we demonstrated that Ch/γ-PGA NPs induced alterations in IL-10-treated macrophages profile, leading to a decreased surface expression of CD163, a monocyte/macrophage specific marker expressed predominantly on cells which exhibit strong anti-inflammatory potential [57]. This alteration was accompanied by a significant increase of TNF-α, IL-6 and IL-12p40 pro-inflammatory cytokines. Altogether, these results suggest that Ch/γ-PGA NPs efficiently modulate macrophages towards a more immunostimulatory profile. These results contrast with the effect of Ch films (DA 11%) on human macrophages [42], but the contact surface and formulation of the polymer highly influence the immune response. In fact, Barbosa *et al.* demonstrated that DA of Ch influences the immune response to Ch scaffolds [58]. Ch scaffolds with DA 15% induced a M1-like response, while Ch scaffolds with DA 5% led to a M2-like response [59]. On the other hand, Oliveira *et al.* showed the *in vitro* effects of Ch films (DA 11%) on cultured human macrophages and concluded that they modulated macrophage polarization towards an M2-like phenotype [42]. These studies highlight the importance of acetyl and amine functional groups and formulation of Ch on driving the inflammatory response.

Recently, iron oxide NPs were described to reduce growth of subcutaneous adenocarcinomas in mice by inducing a local pro-inflammatory macrophage profile [28]. Others, using an experimental model of arthritis, efficiently repolarized M1-like macrophages into M2-like by using tuftsin-coated alginate NPs containing IL-10 plasmid DNA [60]. Cationic dextran and polyethyleneimine were demonstrated to induce a shift in MDSCs profile, through TLR4-mediated signaling, and to reduce subcutaneous tumor growth [61]. Despite several attempts to develop safe and efficient strategies to re-educate TAMs, further studies elucidating the underlying mechanisms and involving more representative cancer models, as orthotopic mice models, are needed. More recently, other macrophage-centered anticancer strategies, which consist in inhibiting their recruitment, suppressing their survival, exploring the blockade of TAMs tumor-promoting activities or promoting the macrophage antitumor effector functions, namely antibody-dependent cellular cytotoxicity and phagocytosis (reviewed in [22,23,62]) are under investigation and entering in

clinical trials. Although these macrophage-targeting approaches might, *per se*, result in some clinical benefits, the combination with conventional therapies and immunotherapies are certainly crucial for a successful outcome.

One of our main interests was to investigate the functional consequences of APCs activation induced by Ch/γ-PGA NPs, which may have implications for anticancer therapies. Since T cell activation plays a central role in antitumor immunity, the capacity of DCs and macrophages to stimulate allogeneic T lymphocytes was evaluated. Ch/γ-PGA NPs-stimulated DCs were able to induce a significant increase of CD4 T cell proliferation, while IL-10-stimulated macrophages treated with Ch/γ-PGA NPs induced a significant increase of CD8 T cells. This differential T cell activation can be explained by the differences in HLA-DR expression on macrophages and DCs. Contrary to what we observed in macrophages, both LPS and NPs induced HLA-DR expression on DCs, leading to CD4 T cell activation when in co-cultures. Furthermore, our results demonstrated enhanced IFN-γ production by cocultures of T cells with NPs-stimulated DCs or NPs-stimulated IL-10-macrophages, suggesting the promotion of pro-inflammatory responses. Altogether, Ch/γ-PGA NPs can modulate DC and macrophage function and ultimately enhance T cell response at different levels.

Uto *et al.* have reported that γ-PGA NPs-treated $CD8^+$ DCs stimulate allogeneic T cells in a dose-dependent manner, *in vitro* [54]. In mouse models, these NPs combined with listeriolysin or ovalbumin induced CD8T cell response [53,54]. Altogether, these results emphasize a clear advantage of our Ch/γ-PGA NPs which, without antigens, potentiate the immune response, and given their efficient drug delivery properties, can be combined with other immunostimulatory molecules.

In the present study, we demonstrated the functional impact of Ch/ γ-PGA NPs on macrophage/DC-mediated T cell proliferation using a heterologous setting. Nevertheless, future experiments should be performed to evaluate the potential ability of Ch/γ-PGA NPs to induce a specific antigen response in an autologous tumor context. Having characterized the impact of Ch/γ-PGA NPs on DCs and macrophages, both phenotypically and functionally, we were interested on the consequences of this activation on CRC cancer cell invasion, as an indirect measurement of macrophage and DCs phenotype modulation. TAMs, known for their similarity with M2-like macrophages, are high producers of pro-invasive and pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), EGF and MMPs [9]. The protumoral role of TAMs in cancer is further supported by clinical studies which found a correlation between the high macrophage infiltration in tumors and a poor patient prognosis [11]. Considering, the protumoral role of IL-10-stimulated macrophages (M2-like) as a functional phenotypic marker [43], we investigated the ability of Ch/γ-PGA NPs to counteract M2-like macrophages-mediated cancer cell invasion. Ch/γ-PGA NPs were indeed able to reduce macrophage-mediated cancer cell invasion, likely related with a more immunostimulatory profile of these macrophages. Regarding DCs, we reported, for the first time, that immature $CD11c^+$ DCs induce RKO cancer cell invasion. Additionally, Ch/γ-PGA NPs had an inhibitory effect on this pro-invasive capacity of DCs. Since DCs within some tumors are described as being immature or dysfunctional, this finding highlights the importance of therapies to promote DC activation at the tumor microenvironment.

Overall, our results demonstrate that Ch/γ-PGA NPs are able to phenotypically and functionally modulate macrophages and DCs, enhancing their capacity to promote T cell proliferation and reducing their capacity to induce CRC cell invasion, constituting therefore a promising solution for the combination of immunomodulatory and conventional anticancer therapies. Since Ch and γ-PGA are attractive adjuvants for injectable and mucosal vaccines, several reports have pointed these biomaterials for therapeutic approaches [45,54,63,64]. Thus, we believe that Ch/γ-PGA NPs present a great opportunity to combine their adjuvant properties with their potential as a drug delivery system for the administration of immunomodulatory cytokines, such as GM-CSF, IL-12 or IFN-γ.

5. Conclusion

In our study, we addressed the use of Ch/γ-PGA NPs as an immunostimulatory anticancer therapy, demonstrating that these NPs had a prominent effect in inhibiting M2-like macrophage polarization and in inducing an immunostimulatory DCs phenotype, *in vitro*, favoring T cell proliferation and inhibiting CRC cell invasion. Their future combination with other immunostimulatory agents will be beneficial for the targeted treatment of cancer via immune response modulation.

Acknowledgements

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Disclosure Statement

The authors confirm that there are no known conflicts of interest associated with this publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.09.016.

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Supplementary Data:

Supplementary Methods

S1.1. Transmission electron microscopy of Ch/γ-PGA NPs

Transmission electron microscopy observations were performed using a Jeol JEM 1400 electron microscope. Briefly, a drop of the NPs suspension was placed onto a 400-mesh copper grid. After 2 min of deposition, the grid was tapped with a filter paper and negatively stained with uranyl acetate. TEM micrographs were obtained with a magnification of 30,000x.

S1.2. Fluorescent Ch/γ-PGA NPs preparation

Fluorescent NPs were prepared according to Gonçalves et al. [34]. First, Ch was labeled with fluorescein isothiocyanate (FITC) to obtain a polymer with a final degree of modification of 5% (5% of Ch amine groups with FITC). 100 mg of dried Ch were dissolved in 100 mL of 1% v/v AcOH at 4ºC until complete dissolution. FITC (11 mg to achieve 5% modification) was dissolved in 100 mL of methanol. Both solutions (Ch and FITC) were mixed at constant stirring, protected from light, for 3 h. The FITC-labeled Ch (ftCh) was then precipitated with 0.5 M NaOH and washed with Milli-Q water until no fluorescence was detected in the supernatant. ftCh was lyophilized, dried and weighted. ftCh/γ-PGA NPs (ftNPs) were prepared as described above.

Supplementary Figures

Supplementary Figure S1

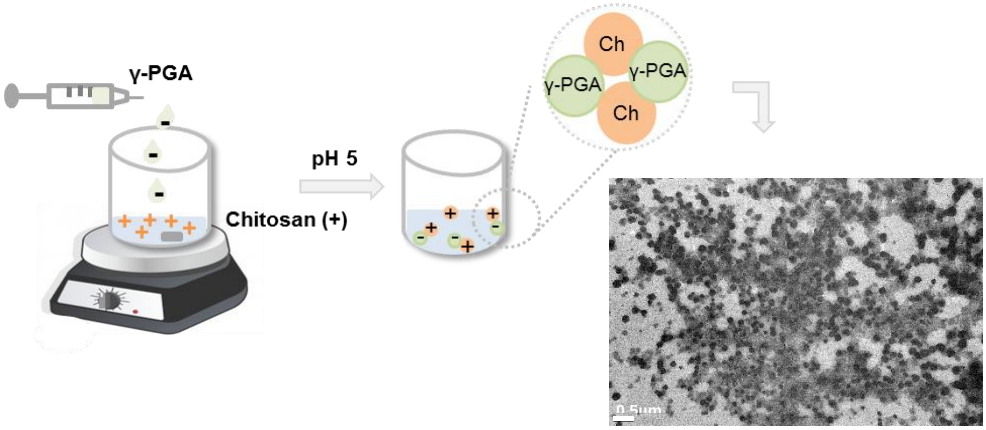

Figure S1. Schematic representation of Ch/γ-PGA nanoparticles (NPs) preparation. NPs were obtained by complex co-acervation method, driven by electrostatic interactions between chitosan and poly(γ-glutamic acid) (γ-PGA) at pH 5. γ-PGA solution was dropped to Ch solution under high stirring at room temperature, as previously described [33]. Ch/γ-PGA NPs morphology was observed using a Jeol JEM 1400 transmission electron microscope (TEM). Samples were prepared by placing a drop of the NPs suspension onto a 400 µl mesh copper grid coated with carbon. After 2 min of deposition, the grid was tapped with a filter paper and negatively stained with uranyl. TEM images were obtained with a magnification of 30,000x. Scale bar: 0,5µm.

Supplementary Figure S2

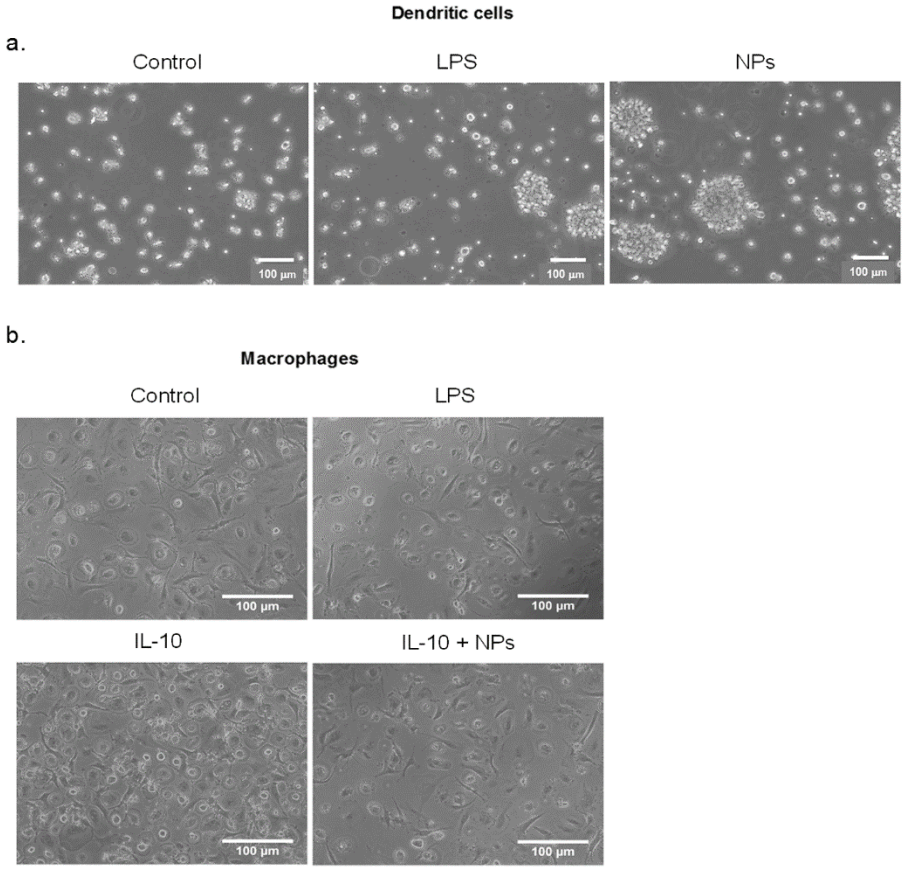

Figure S2. Cell morphology of DCs and macrophage cultures. Brightfield microscopic images of **a.** Differentiated DCs stimulated or not with LPS or Ch/γ-PGA NPs; **b.** differentiated macrophages stimulated or not with LPS, IL-10 or with IL-10 together with Ch/γ-PGA NPs, at day 7 and day 13, respectively. Scale bar represents 100µm in DCs and macrophages images.

Supplementary Figure S3

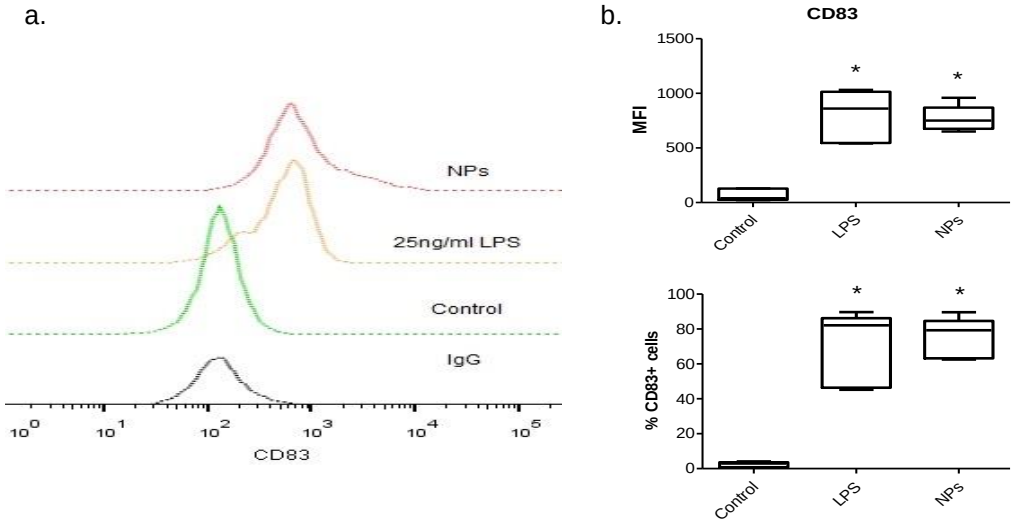

Figure S3. Ch/γ-PGA NPs induce DC maturation. Differentiated DCs stimulated with LPS or Ch/γ-PGA NPs for 48 h were surface stained for the lineage marker CD11c and the maturation marker CD83 and analysed by flow cytometry. **a.** The histogram of a representative phenotypic profile shows the electronic gate used to identify $CD83^+$ cells gated on single cells. **b.** The MFI of CD83 was determined by subtracting the fluorescence intensity of respective isotype control and the percentage of positive cells was determined. Data is representative of at least 5 donors and median is represented by the horizontal line inside the box plots. Comparisons were performed using the Friedman test followed by Dunn's multiple comparison test (* $p<0.05$).

Supplementary Figure S4

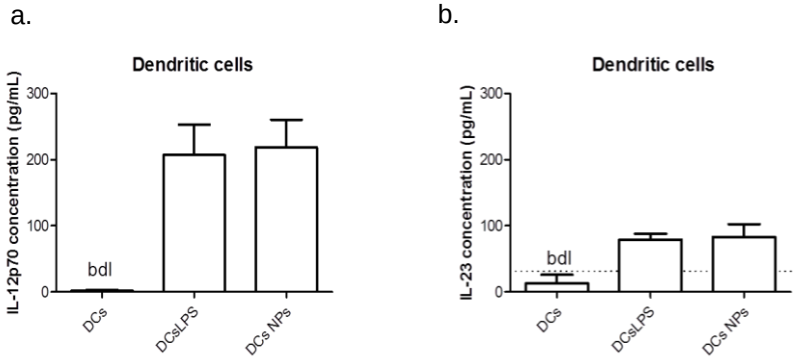

Figure S4. Ch/γ-PGA NPs induce higher production of IL-12Pp70 than IL-23 by DCs. Conditioned media from DCs stimulated with LPS or NPs were recovered and the levels of the IL-12p70 and IL-23 were determined by ELISA. Data is representative of at least 4 different blood donors. Comparisons were performed using the Friedman test followed by Dunn's multiple comparison test relative to unstimulated macrophages (* $p<0.05$).

Supplementary Figure S5

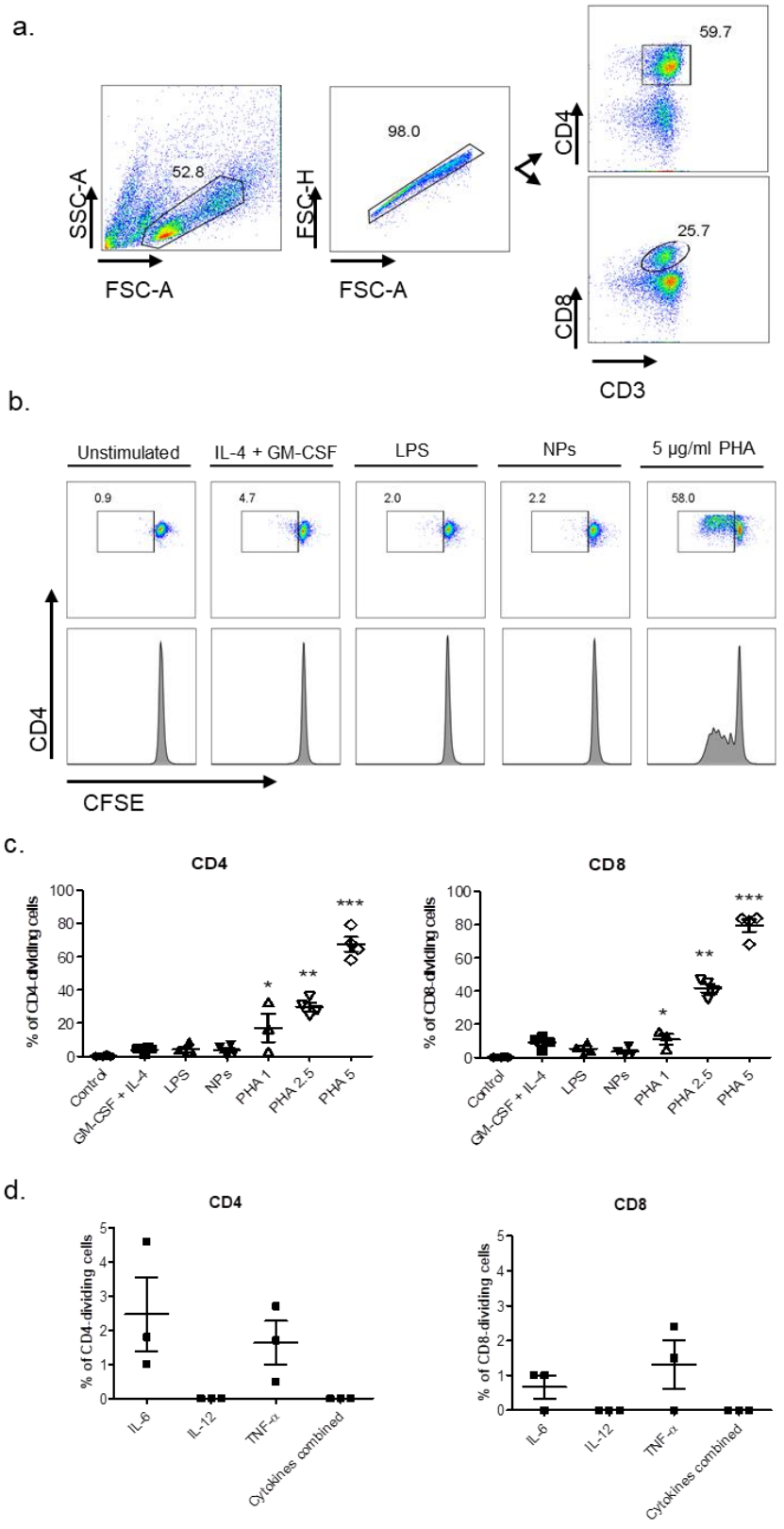

Figure S5. T cell proliferation analysis. Lymphocytes isolated from a different blood donor labeled with allogeneic CFSE were cultured with DCs differentiating cytokines (GM-CSF and IL-4), PHA, LPS, NPs and PHA as experimental controls. After 7 days of co-culture, cells were surface stained for CD3, CD4 and CD8 followed by flow cytometry analysis. **a.** CD3 vs CD4 and CD3 vs CD8 from the gated single cells show the populations corresponding to T helper cells ($CD3^+$ $CD4^+$) and to cytotoxic T cells ($CD3^+$ $CD8^+$). Numbers indicate the percentage of the previously cited populations. **b.** Representative CD4 vs CFSE plots of the experimental controls performed during MLR assay. **c.** Percentage of $CD4^+$ and $CD8^+$ dividing cells were determined. Comparisons were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test (* $p<0.05$; ** $p<0,01$; ***$p< 0.001$). **d.** CFSE-Lymphocytes were also stimulated with IL-6 (10 ng/mL), IL-12 (2 ng/mL) or TNF-α (1.5 ng/mL) or all three cytokines combined for 6 days. Then, cells were surface stained as described above. Percentage of $CD4^+$ and $CD8^+$ dividing T cells were determined. Data of 4 donors of lymphocytes are represented. Data were compared by Friedman test followed by Dunn's multiple comparison test and no statistical significant differences were found.

CHAPTER V

Chitosan/poly(γ-glutamic acid) nanoparticles as immune adjuvants to radiotherapy

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Manuscript in preparation

ABSTRACT

Radiotherapy not only exerts direct effects on tumor cells, but also reprograms the tumor microenvironment to elicit an effective immune response. However, radiation can attract immunosuppressive cells into the tumor microenvironment opening the perspective to combinatorial treatments with immunotherapies. In fact, previous studies from our group showed that ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype, preserving still their pro-invasive and pro-angiogenic abilities. Considering the well described functional plasticity of macrophages, these cells are appellative targets for anticancer strategies. We have recently reported that chitosan (Ch)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) modulate immature/immunosuppressive antigen-presenting cells (APCs) towards an immunostimulatory profile, impairing APCs ability to induce cancer cell invasion. Therefore, the immunostimulatory properties of Ch/γ-PGA NPs suggest them as good candidates as adjuvants to anticancer therapies.

Here, we addressed the synergistic potential of Ch/γ-PGA NPs to be combined with radiotherapy (RT) in immunocompetent 4T1 orthotopic breast tumor mouse model. Therefore, animals were divided in four groups: non-treated, treated with NPs, with RT or with the combination of both treatments (NPs+RT). Tumor burden, lung metastasis formation and immune cell profile were explored.

Non-treated animals had progressive tumor growth and developed lung metastasis. NPs-treated animals had a negligible effect on tumor progression, while RT-treated decreased primary tumor burden. When both treatments were combined, breast tumor growth was further impaired. Notably, RT+NPs treatment potentiated the NPs effect in 46% and RT in 30% and, reduced tumor growth in 56% comparing to non-treated animals. Despite all groups presented a similar bioluminescence signal in the lungs, animals from the NPs+RT group presented less and smaller metastatic foci, in comparison to control or single treatments. Systemically, the protumoral cytokines IL-4, IL-10 and the chemokine CCL4 were significantly decreased in the combinatorial treatment. Overall, these results suggest that Ch/γ-PGA NPs potentiate and synergize with RT, opening new perspectives to be used in anticancer strategies.

1. INTRODUCTION

Radiotherapy is an effective and broadly used treatment for several solid cancers, including breast, prostate, cervical, head and neck cancers [1]. Its biological principle is based on the induction of DNA breaks on aberrant proliferative and DNA-repair-deficient cells. Consequently, cell death releases tumor-associated antigens into the tumor microenvironment as well as several damage-associated molecular patterns, eliciting a systemic antitumor immune response [2, 3]. Recent advances in the radiobiology field demonstrated that tumor-associated stromal cells impact the radiotherapy outcome [4]. Currently, it is known that tumor irradiation induces a wound healing response characterized by inflammation, extracellular matrix remodeling and infiltration of immunosuppressive myeloid cells, which may facilitate tumor resistance and relapse. The abundant myeloid immune infiltrate at the irradiated tumors consists of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), likely recruited to limit tissue damage induced by radiotherapy, also contributes to dampen the antitumor immunity and escape from immunosurveillance [5-7]. In fact, the accumulation of myeloid cells, including MDSCs and TAMs, have been associated with tumor progression and metastasis [8-10]. The blockade of TAMs recruitment, achieved by CSF1R inhibitors, has been associated with improved responses to therapy in preclinical studies [11] but no significant clinical benefits were yet observed [12]. Most recently, Kumar and co-workers demonstrated that the recruitment of cancer-associated fibroblasts to the tumor site limits CSF1R targeted therapy, thus highlighting the importance of combining CSF1R blockade with CXCR2 inhibitors [13]. Once the functional plasticity of TAMs and granulocytic cells and the differential impact on tumor progression is well established, the reprogramming of these cells appears as more appellative therapeutic strategy rather than deplete them [14, 15] and may potentiate the radiotherapeutic effects.

Our team has previously explored the effect of clinically relevant ionizing radiation doses (5*2Gy) on human macrophage function and on macrophage-cancer cells crosstalk. It was demonstrated that besides the induced DNA damage, irradiated macrophages remain viable and metabolically active, most probably through the pro-survival NF-kB signaling pathway activation. Additionally, ionizing radiation drove macrophages towards a more pro-inflammatory profile but conserved their ability to induce cancer cell invasion and angiogenesis [16]. Considering the infiltration of macrophages in the post-radiotherapy microenvironment, there is an opportunity to target these cells following radiotherapy treatment and restore T cell-mediated antitumor immune response.

Biomaterials have been widely studied for vaccine delivery, once they could protect antigen and adjuvant molecules from degradation, increase lymphoid organ accumulation, and modulate antigen-presenting cells (APCs) functions. In fact, several biomaterial-assisted cancer vaccines have shown great potential in preclinical and clinical development [17-22]. For example, synthetic high-density lipoprotein (sHDL) nanodiscs efficiently co-delivered tumor antigens and the adjuvant CpG to draining lymph nodes, and promoted dendritic cells (DCs) maturation, eliciting cytotoxic T lymphocyte (CTL) responses. The combination with immune checkpoint inhibitors further amplified the potency of nanodisc vaccination, leading to elimination of established tumors [18]. Others showed that nanodiscs combined with multiple TLR agonists were strong adjuvants for vaccines [23]. We have recently reported that chitosan (Ch)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) modulated immature DCs towards an immunostimulatory profile, eliciting CD4 T cell response. Furthermore, Ch/γ-PGA NPs reprogrammed IL-10-stimulated macrophages, characterized by an immunosuppressive and protumoral phenotype, towards an immunostimulatory one, potentiating CD8 T cell response. Notably, Ch/γ-PGA NPs hindered the ability of macrophages and DCs to induce colorectal cancer cell invasion [24]. Additionally, Ch/γ-PGA NPs were appointed as potential carriers for immunomodulatory drugs [25]. Therefore, the combination of immunostimulatory and delivery properties of Ch/γ-PGA NPs make them good candidates as adjuvants for anticancer therapeutic strategies.

Thus, the aims of the present work were to evaluate the ability of Ch/γ-PGA NPs to synergize with radiotherapy treatment to (1) control tumor progression, (2) inhibit lung metastasis, and (3) reprogram the immune response. This report highlights the potential of Ch/γ-PGA NPs to be combined with radiotherapy as an anticancer therapeutic strategy, based on their immunostimulatory properties and ability to control breast tumor progression and lung metastasis formation.

2. MATERIAL AND METHODS

2.1. Ch/γ-PGA NPs preparation and characterization

Ch/γ-PGA NPs were prepared as previously described [24]. Briefly, Ch (France-Chitine) with the degree of acetylation (DA) of 10.4 ± 1.6%, determined by Fourier transform infrared spectroscopy using KBr pellets (FTIR-KBr), and molecular weight (MW) of 324 ± 27 kDa, determined by size-exclusion chromatography, was used. γ-PGA, with MW of 10-50 kDa and a purity level of 99.5%, was produced from *Bacillus subtilis* cultures, as described by Pereira *et al* [26]. Ch/γ-PGA NPs were prepared at a molar ratio of 1:1.5 (mol Ch:mol γ-PGA) by a co-acervation method. γ-PGA solution (0.2 mg/mL in 0.05 M Tris-HCl with 0.15 M NaCl buffer solution) was dropped to a Ch solution (0.2 mg/mL in 0.2 M AcOH), using a 1 mL syringe copulated to a syringe pump (KD Scientific Inc., Holliston, MA), at constant speed (3.6 µL/s), under high stirring conditions and at room temperature (RT). All the solutions were prepared in buffer solution with pH adjusted to 5.0. After initial preparation, Ch/γ-PGA NPs were concentrated 10 times by centrifugation (13000 rpm, 30 minutes (min), 4 ºC). Ch/γ-PGA NPs size, polydispersion index and zeta potential was then evaluated by dynamic light scattering (DLS) in a ZetaSizer Nano Zs (Malvern Instruments), equipped with a He-Ne laser (λ=633 nm). Each NPs batch produced was tested and 3 measurements per sample were acquired.

2.2. Cell lines

The 4T1 cell line expressing luciferase (4T1-Luc), a mouse triple negative breast cancer cell line, was obtained from Sibtech (Brookfield, CT, USA). Tumor cells were defrosted and maintained/expanded in Dulbecco's Modified Eagle's Medium (Gibco; Life Technologies), supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco; Life Technologies) and 1% (v/v) penicillin/streptomycin (Sigma) (cDMEM) at 37°C and 5% CO_2 humidified atmosphere. All cultures were regularly tested for Mycoplasma by using MycoAlert Plus Kit (Lonza, Basel, Switzerland).

2.3. Ethics Statement

All animal experiments were performed in strict accordance with the recommendations of the European Union Directive 2010/63/EU and the Helsinki declaration, following a protocol previously approved by the UZ-Ghent University Hospital Ethics Committee (ECD 17/124). At the defined endpoints, mice were euthanized by cervical dislocation by trained personal, and certified by the Portuguese Direção-Geral de Alimentação e Veterinária to minimize suffering.

2.4. Animal studies

Four-week-old female immunocompetent BALB/cByJ (Charles River Laboratories, l' Arbresle Cedex, France) were injected orthotopically in the mammary fat pad with $1x10^6$ 4T1-Luc cells, resuspended in 100 µl serum-free DMEM in Matrigel matrix (1:1) (Corning). Tumor implantation was confirmed by bioluminescence imaging after 24 hours (h) of tumor cell injection. Mice were intraperitoneally injected with 150 mg/kg body weight D-luciferin (Caliper Life Sciences), 20 min before bioluminescence imaging, which was carried out by using an IVIS Lumina II (Caliper Life Sciences). Bioluminescent imaging was initiated by a cooled CCD camera in the IVIS with a 15-cm field of view, binning factor of 8, 1/f stop and open filter. Exposure times were set automatically, according to the luciferase signaling activity. Regions of interest (ROIs) were drawn for primary tumor and metastatic sites and were calculated through the IVIS software, expressed in total flux (photon/s). After seven days of tumor cell inoculation, when the tumors reach around 50 mm^3, animals from radiotherapy (RT) and combinatory therapy (RT plus Ch/γ-PGA NPs) groups were locally irradiated with hypofractionated 10 Gy (2x5 Gy) using a Small Animal Radiation Research Platform, SARRP system (X-ray tube: ISOVOLT 225M2 X-ray source; SARRP system, XStrahl®, Surrey, UK), at a constant rate of 2.83 Gy/min, for 106 seconds. Ionizing radiation experiments were performed at day 7 and 9. The voltage of the X-ray source is fixed at 220 kV with a tube current of 13 mA, emitted from the 2.5 mm focal spot and filtered by a copper filter of 0.15 mm and a 5 mm × 5 mm collimator. After 3 days, animals from Ch/γ-PGA NPs and RT plus Ch/γ-PGA NPs groups were treated subcutaneously near the tumor, 6 times for 2 weeks with Ch/γ-PGA NPs (0.7 mg/mL). Tumor volume was measured using a caliper and calculated as (length × width × width)/2 (mm^3) and tumor progression was followed by bioluminescence imaging every week. Animals were sacrificed at day 28.

2.5. Spleen digestion and stimulation

Spleens were collected 4 weeks after tumor cell inoculation and were mechanically digested. Erythrocytes were lysed with red blood cell lysis buffer (Biolegend) for 3 min at RT. Spleen cells for myeloid staining were stimulated with 1 µg/mL of LPS (Sigma) and 10 ng/mL of recombinant mouse interferon-gamma (Peprotech) for 4 h at 37 °C. For lymphoid staining, tumor cells were stimulated with Phorbol 12-myristate (PMA) (200 ng/mL, Sigma), ionomycin (1 µg/mL, Sigma) and Brefeldin A (BFA) (10 µg/µL, Sigma), for 4 h at 37 °C.

2.6. Flow Cytometry

After stimulation, cells were washed in FACS buffer (PBS, 2% FBS, 0.01% sodium azide) and pre-treated with Fc blocking agent (anti-mouse CD16/CD32, Biolegend) for 10 min to

minimize non-specific antibody binding. Then, cells were stained in the dark, for 45 min at 4 ºC, with specific conjugated antibodies, as: CD3-PerCPcy5.5 (clone145-2C11, eBioscience), CD4-BV421 (clone RM4-5, Ebioscience), CD8 (clone 53-6.7, Biolegend), CD11b-Alexa 700 (clone M1/70, Biolegend), CD11c-FITC (clone N418, Biolegend), CD45-BV510 (clone 30-F11, Biolegend), CD45-APC (clone 30-F11, Biolegend), CD206-PerCPcy5.5 (clone C068C2, Biolegend), Ly6C-BV605 (clone HK1.4, Biolegend), Ly6G-BV421 (clone 1A8, Biolegend), F4/80-PEcy7 (clone BM8, Ebioscience) and MHC II-APCcy7 (clone M5/114.15.2, Ebioscience). The Aqua Zombie-BV510 and Live Dead-APC-Cy7 were used to confirm cellular viability. After staining, cells were washed and fixed with 2% of paraformaldehyde overnight. Then, cells were permeabilized with Permeabilization buffer (Invitrogen), in the dark, for 20 min at RT. Then, cells were intracellular stained for intracellular antigens, for 30 min at RT, using the following antibodies: IL-17-FITC (clone TC11-18H10, Biolegend), IFN-γ-BV605 (clone XMg1.2, Biolegend), TNF-α-PE (clone MP6-XT22, Ebioscience) and FoxP3-APC (clone FJK-16S, Ebioscience). Isotype-matched antibodies were used as negative controls. After additional washes, cells were acquired on a FACS Canto Flow Cytometer (BD Biosciences) and BD FACSDiva software. Results were analyzed using FlowJo software version 10 (TreeStar, Inc.).

2.7. Serum collection and analysis

Blood was collected from all animals by cardiac puncture to non-coated eppendorfs. Then, blood samples were centrifuged at 2500 rpm, for 30 min at RT. The serum fraction collected was centrifuged to remove erythrocytes at 1200 rpm, 5 min, at 4 ºC and stored at -80 ºC until use. The samples were analyzed for cytokines and chemokines using a mouse cytokine array/chemokine array 31-multiplex (MD31) through Eve Technologies services.

2.8. Histology

Mice breast primary tumors, lungs, liver, spleen and the left kidney were maintained for 24-48 h in formalin at RT. After, these organs were embedded in paraffin, sectioned into 3 µm thickness sections, and stained with haematoxylin and eosin.

2.9. Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software version 7 (GraphPad-v7). Due to the limited number of donors included in test groups ($n \leq 7$), a non-Gaussian distribution of the data was considered for the statistical analysis. Therefore, the non-parametric unpaired Kruskal Wallis test was used for non-paired comparisons between groups. Statistical significance was achieved when *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3. RESULTS

3.1. Ch/γ-PGA NPs in combination with RT treatment do not compromise animals safety

The murine mammary 4T1 model is well described in the literature [27, 28] and its poorly immunogenicity, spontaneously metastatic and highly malignant condition in syngeneic mice make it an attractive model to experiment new anticancer therapies. Our previous *in vitro* data described that radiotherapy (RT) modulates macrophages towards a pro-inflammatory phenotype, sustaining still their ability to promote cancer cell invasion [16]. Recently, we developed Ch/γ-PGA NPs and confirmed their immunomodulatory properties on macrophages and DCs with impact on T cell proliferation [24]. Considering this recent knowledge, in the current study, we explored the potential of Ch/γ-PGA NPs alone and as adjuvants to RT. Thus, 4T1 cells-expressing luciferase (4T1-Luc) were injected subcutaneously on the mammary fat pad of BALB/cByJ mice. At the seventh and tenth day after tumor inoculation, animals from groups RT and RT+NPs were locally irradiated with hypofractionated 10 Gy (2*5 Gy) using the SARRP platform. After 3 days, animals from RT+NPs or NPs group were boosted with local subcutaneous Ch/γ-PGA NPs, three times/week (Fig. 1a). Along the experiment, animal body weight was monitored every week and no alterations were observed in any of the treatment conditions (Fig. 1b). At the end of the experiment, at day 28, liver and kidneys were harvested to evaluate the impact of possible accumulation of NPs in these organs. Hematoxylin-eosin staining revealed no morphological differences in the liver and kidneys from non-treated (control, CTR) and treated (RT, NPs and RT+NPs) animals (Fig. 1c), ensuring that these experimental conditions do not compromise animal safety.

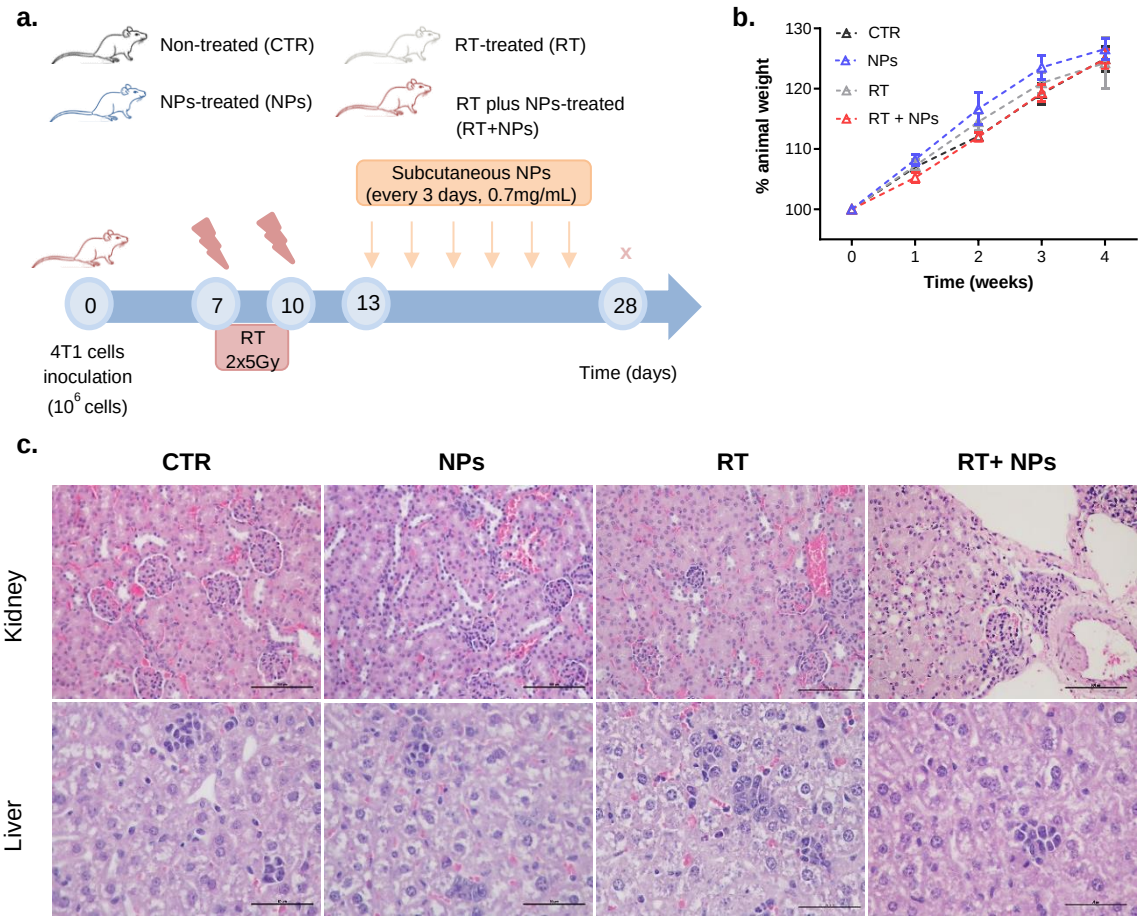

Figure 1. Ch/γ-PGA NPs plus RT do not impact animal weight neither liver or kidney morphology. BALB/c mice injected with 4T1 cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5 Gy) or treated with Ch/γ-PGA NPs (NPs), or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. **a.** Experimental timeline. Animals from RT and RT+NPs group were irradiated at day 7 and day 10 with 2*5 Gy. After 3 days, animals from NPs and RT+NPs group were primed with Ch/γ-PGA NPs three times/week for 2 weeks. **b.** Animal body weight was evaluated every week during 5 weeks. Time 0 corresponds to the week before tumor cells inoculation. **c.** Mice´s liver and kidney were processed for histological procedures and stained with hematoxylin and eosin. Kidney images: magnification 200x; scale bar: 100 µm. Liver images: magnification 400x; scale bar: 50 µm. Data show the mean ± SEM and it is representative of at least 7 animals. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test relative to non-treated control or single treatments (NPs or RT). No significant differences were found.

3.2. Ch/γ-PGA NPs in combination with RT treatment significantly decrease the 4T1 primary tumor progression

RT is a localized treatment highly effective in killing primary tumor cells localized within the field of the radiation beam. Dying cells release tumor-associated antigens and danger signals which activate innate immune cells, eliciting T cell activities. However, immunosuppressive cells recruited to the irradiated tumor might compromise the triggering of an effective antitumoral T cell response [29]. Thus, we asked if Ch/γ-PGA NPs administration alone or in combination with RT decrease breast tumor progression. Therefore, we followed primary tumor growth through tumor volume measurements and bioluminescence imaging. Our results evidenced that tumors from non-treated animals grew progressively while tumors from treated animals experimented alterations in such progression pattern (Fig. 2a). While the effect of NPs on tumor progression was negligible (431.8 ± 78.7 %) comparing to control animals (524.7 ± 80.2 %), the RT treatment delayed tumor progression (333.4 ± 69.1 %), although without reaching statistical significance ($p<0.09$). Importantly, the combination of RT with NPs administration resulted in a significant decrease of tumor growth (233.6 ± 22.2 %, $p<0.003$), potentiating the effect of the single treatments with NPs in 46 % and of RT in 30 % (Fig. 2b). In agreement, we observed that tumors from control animals presented higher weight (0.7 ± 0.2 g) comparing to treated animals. While NPs-treated animals presented a slight decrease in tumor weight (0.5 ± 0.2 g), the RT treatment significantly reduced tumor weight (0.4 ± 0.1 g). Once again, the combinatorial treatment of RT+NPs further decreased the tumor weight (0.3 ± 0.2 g), although not reached statistical significance to each of the single treatments (Fig. 2c). These results were corroborated by bioluminescence imaging, being the tumors treated with the combinatorial treatment the ones presenting lower bioluminescence, suggesting lower tumor burden (Fig. 2d). The quantification of bioluminescence through total photon flux (photons/second, p/s) at the tumor region showed that NPs slightly impacted tumor burden ($(7.8 \pm 1.4)x10^7$ p/s) comparing to control animals ($(13.0 \pm 2.6)x10^7$ p/s). Importantly, RT treatment decreased tumor bioluminescence ($(6.2 \pm 1.1)x10^7$ p/s), although without reaching statistical significance ($p<0.07$), while the combinatorial treatment of RT+NPs significantly reduced tumor burden ($(3.2 \pm 1.7)x10^7$ p/s) (Fig. 2e). In addition, primary tumors processed for hematoxylin and eosin staining and seemed that tumors from NPs and RT+NPs presented higher areas of necrosis comparing to control animals (Fig. 2f), but further quantification of the percentage of necrotic area is currently ongoing. Overall, these results suggest that the combination of RT with NPs better control tumor progression comparing to single-treated animals.

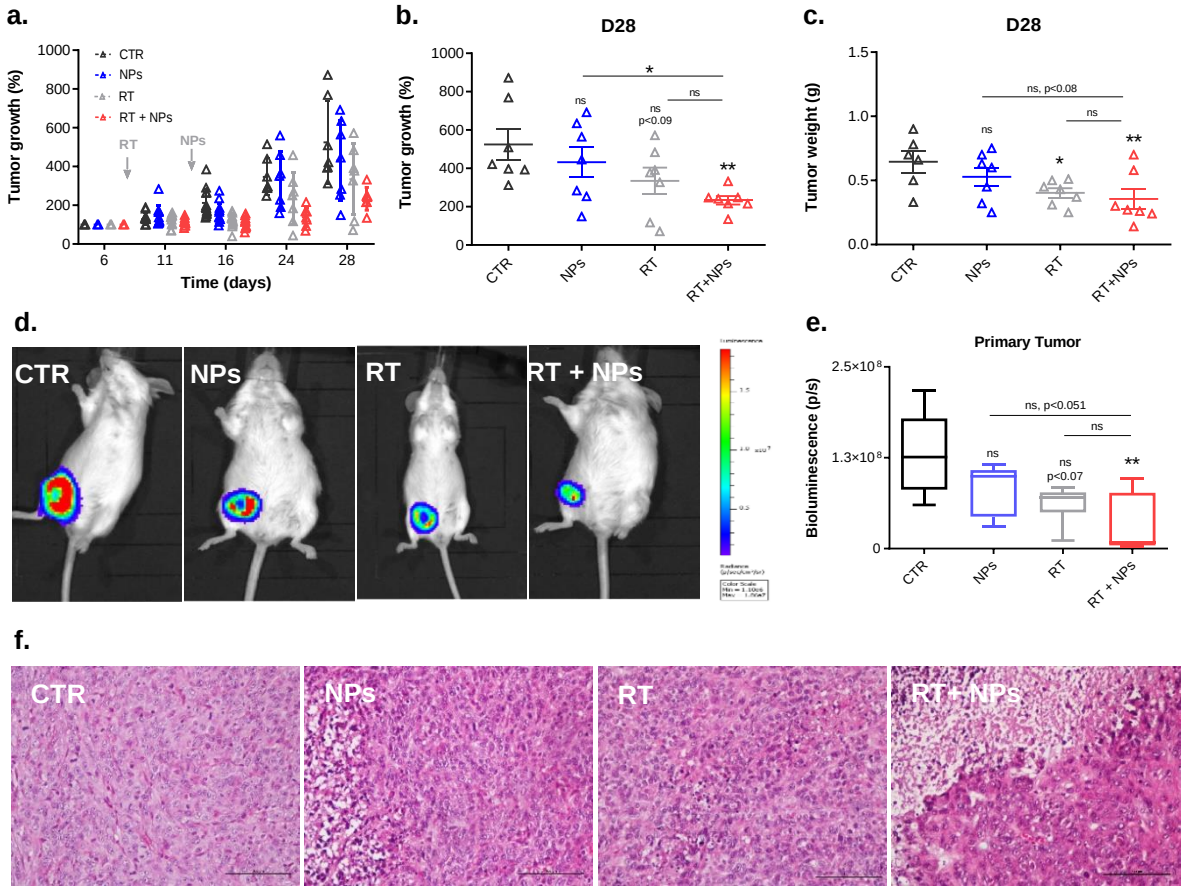

Figure 2. Ch/γ-PGA NPs potentiate RT effects by decreasing 4T1 primary tumor progression. BALB/c mice injected with 4T1 cells expressing luciferase (4T1-Luc) on the mammary fat pad were submitted to radiotherapy (RT) (2x5Gy) or treated with Ch/γ-PGA NPs (NPs), or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. **a.** Tumor volume (mm^3) was measured using a caliper and the growth kinetics were normalized to the initial tumor volume for each animal. Based on this, relative (%) tumor growth was estimated. Values represent the average tumor growth of 7 animals and flags represent standard deviation values. **b. and c.** Relative (%) tumor growth and tumor weight were measured at the end of experiment, at day 28 (D28). Data show the mean ± SEM. **d.** Mice were bioluminescence imaged to monitor tumor progression. Images were taken 20 minutes after intraperitoneal injection of luciferin. Representative images from D28 are illustrated. **e.** Quantification of total photon flux (the number of photons/second, p/s) at primary tumor region. Median is represented by the horizontal line inside the box plots. **f.** Tumors were processed for histological procedures and stained with hematoxylin and eosin, magnification 200x; scale bar: 100 µm. Data is representative of at least 7 animals per group. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^{\#}p<0.05$ and $^{**}p<0.01$ relative to non-treated control or single treatments (NPs or RT)).

3.3. Ch/γ-PGA NPs in combination with RT treatment attenuate spleen leukemic reaction

The 4T1 model was described to induce a leukemic reaction with granulocytosis and splenomegaly following the injection of cells on the mammary fat pad of BALB/c mice and this condition was associated with tumor-derived growth factors [30]. In agreement, we analyzed animals' spleen and observed that spleen weight from control animals had increased 9-fold by four weeks post-tumor transplant comparing to non-tumor induced animals (data not shown). Animals treated with NPs or RT had a negligible decrease of spleen weight (0.7 ± 0.2 g or 0.6 ± 0.1 g, respectively) comparing to control animals (0.9 ± 0.1 g). RT plus NPs treatment present a lower spleen weight (0.3 ± 0.08 g) in comparison with control or single-treated animals, probably due to the inhibition of granulocytic hyperplasia (Fig. 3a,b). These results were corroborated with hematoxylin and eosin staining of spleens. In fact, we observed an exuberant reactive hyperplasia in the spleens of control group animals where the red and white pulp cannot be distinguished, whereas the spleens of those treated with single treatments, NPs or RT, presented a slight decrease in the hyperplasia condition, being more accentuated in the animals treated with the combinatorial treatment RT+NPs (Fig. 2c). These results suggest that the combination of RT with NPs decrease the leukemic condition of 4T1 model. Additionally, we quantified, in the mice serum, the myeloid colony-stimulating factors, namely GM-CSF, G-CSF and M-CSF in the mice serum, previously described to be associated with leukemic reaction in the 4T1 model [30]. A slight reduction of GM-CSF production in animals treated with NPs or with RT+NPs (9.4 ± 3.4 or 8.0 ± 3.6 pg/mL, respectively) in comparison with control animals (33.3 ± 14.4 pg/mL) was observed ($p<0.1$). Interestingly, RT treatment did not impact on GM-CSF levels (39.1 ± 17.9 pg/mL) (Fig. 3d). Regarding G-CSF, control animals had increased 11-fold comparing to non-tumor induced animals (data not shown). NPs- and RT-treated animals presented similar G-CSF levels to the control animals, while the combination of RT with NPs significantly increased G-CSF secretion in the serum (Fig. 3e). No evident alterations were observed in M-CSF levels in the serum of all groups (Fig. 3f). Altogether, RT+NPs treatment impairs leukemic reaction induced by 4T1 cell inoculation, which can be partially mediated through the decrease in GM-CSF and the increase in M-CSF production.

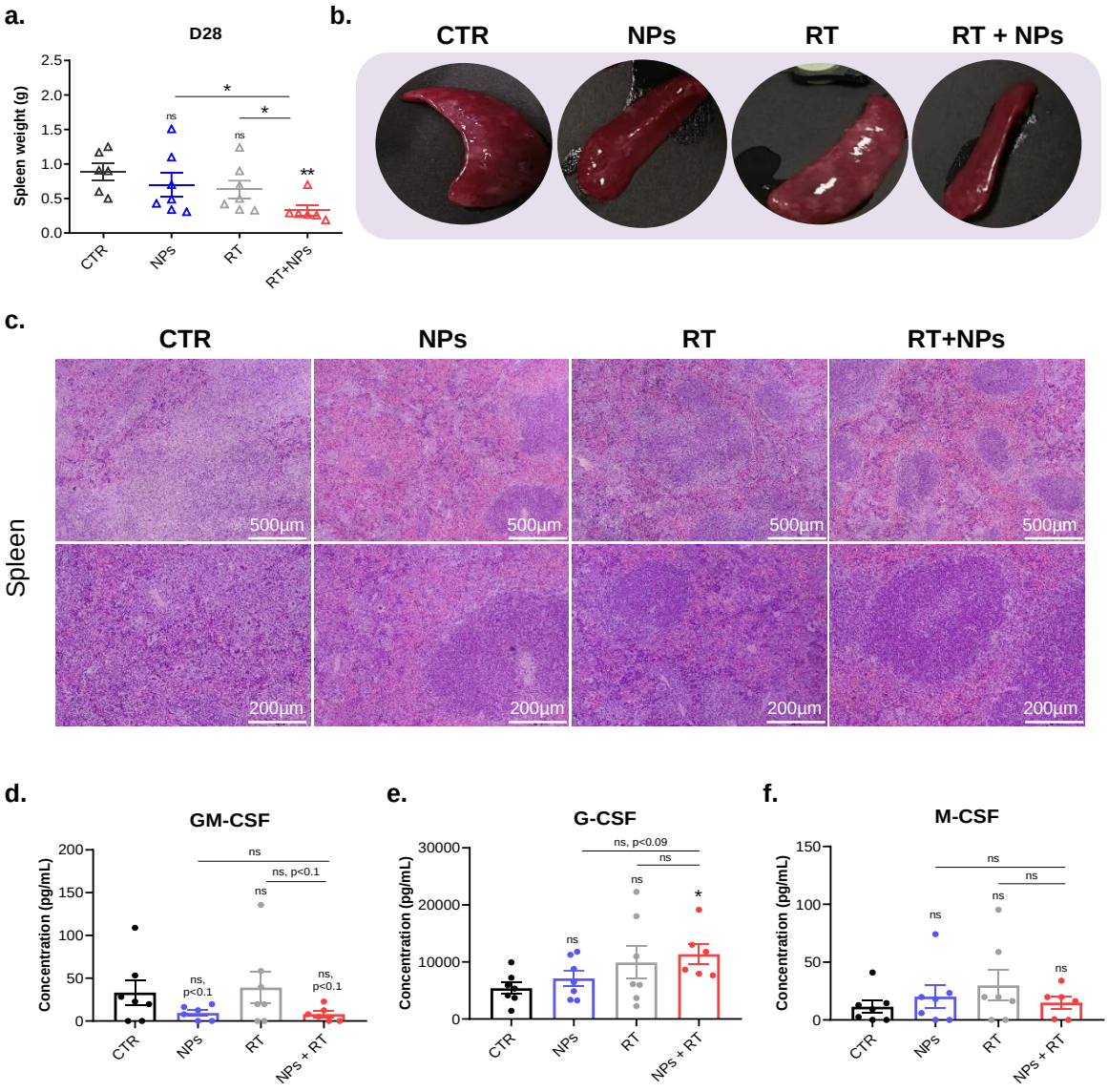

Figure 3. Ch/γ-PGA NPs plus RT decrease spleen weight. BALB/c mice injected with 4T1-Luc cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5Gy), treated with Ch/γ-PGA NPs (NPs), or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. **a.** For each animal, spleen weight (g) was measured at the experimental endpoint, day 28 (D28). Values represent the average spleen weight of 7 animals and flags represent standard deviation values. **b.** Representative images of mice spleen. **c.** Spleens were processed for histological procedures and stained with hematoxylin and eosin, magnification 4x; scale bar: 500 µm; magnification 10x; scale bar: 200 µm. Data is representative of at least 7 animals per group. **d-f.** Serum was analyzed at day 28 (D28) through multiplex immunobead assay technology. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 6 animals. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^{*}p<0.05$ and $^{**}p<0.01$ relative to non-treated control or single treatments (NPs or RT)).

3.4. Ch/γ-PGA NPs in combination with RT treatment increase CD4 T cell response in the spleen

The progression of 4T1 tumors is, generally, accompanied by a decrease of T cells in the spleen and an increase in granulocytes [30]. To understand the impact of the combinatorial treatment on the cellular dynamics of the spleen, we dissociate spleens from different groups at day 28 into single cell suspensions and analyzed, by flow cytometry, for lymphoid populations. Briefly, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were gated on the $CD45^+LD^-$ cells. T cells-producing IFN-γ were gated on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells, while FoxP3-expressing cells were gated on $CD3^+CD4^+$ T cells (Fig. 4a). Interestingly, we observed that NPs and RT treatments had a negligible impact on T cells in the spleen (Fig. 4b-g) whereas the combination of RT with NPs increased the percentage of $CD3^+$ T cells in comparison to the control group (Fig. 4b). This increase was only observed in $CD3^+CD4^+$ T cells (Fig. 4c), which express higher amounts of IFN-γ, comparing to control or RT-treated animals (Fig. 4d). Notably, no alterations were observed in $CD3^+CD4^+$ T cells-expressing FoxP3 (Fig. 4e). The percentage of $CD3^+CD8^+$ T cells in the spleen, at day 28, was reduced comparing to $CD3^+CD4^+$ T cells and no alterations were observed in all treated groups (Fig. 4f). $CD3^+CD8^+$ T cells seemed to express higher amounts of IFN-γ in the spleens of animals treated with RT+NPs (Fig. 4g). Overall, these results suggest that the combination of RT with Ch/γ-PGA NPs increase T helper 1 ($CD3^+CD4^+$IFN-γ^+ cells) response in the spleen, which can be crucial to impair tumor progression. The characterization of myeloid cells is currently ongoing.

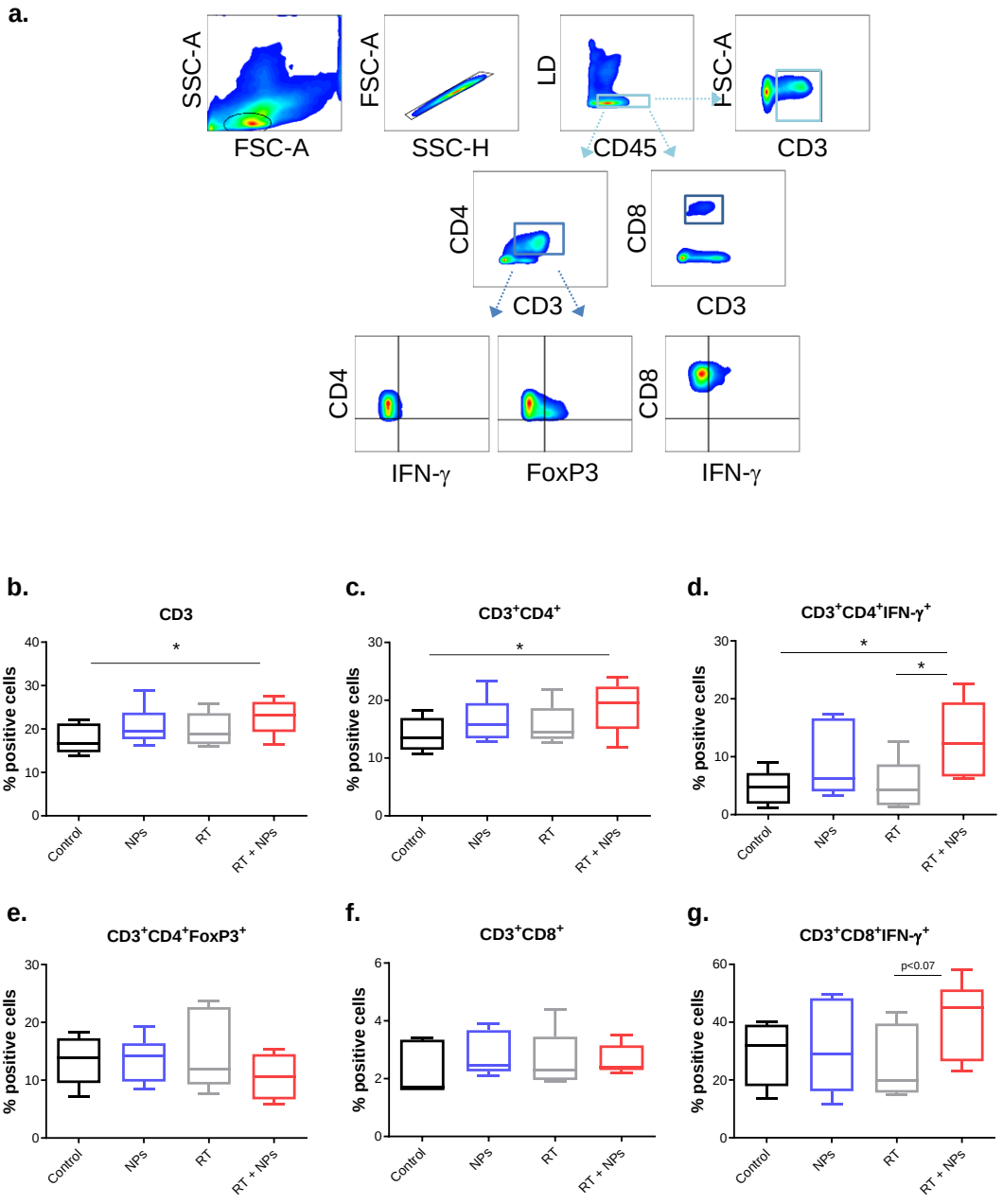

Figure 4. Ch/γ-PGA NPs plus RT increase spleen lymphoid cellular dynamics. BALB/c mice injected with 4T1-Luc cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5 Gy) or treated with Ch/γ-PGA NPs (NPs) or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. Spleens were collected and processed for flow cytometry analysis. **a.** The pseudocolor plots indicate the gate strategy of T cells. $CD45^+$Live Dead $(LD)^-$ cells were gated on single cells (FSC-A vs SSC-H). $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were gated on $CD45^+LD^-$ cells. IFN-γ-producing T cells were gated on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. FoxP3-expressing cells were gated on $CD3^+CD4^+$ T cells. **b-g.** The percentage of positive cells was determined. Data is representative of at least 5 animals. Median is represented by the horizontal line inside the box plots. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^*p<0.05$ relative to non-treated control or single treatments (NPs or RT)).

3.5. Ch/γ-PGA NPs in combination with RT treatment slightly impact the accumulation of T cells at the tumor site

Lymphoid cells are described to exist in lower percentage in 4T1 primary tumors, with their percentage decreasing with tumor progression [27]. Therefore, we investigated if the combinatorial treatment had any impact on the dynamics of lymphoid cells at the primary tumor site. Importantly, we observed no significant alterations in the percentage of T cells present at the primary tumor (Fig. 5a-f). In animals treated with RT+NPs, a slight increase was observed on $CD3^+CD4^+$ T cells ($p<0.1$) and on $CD3^+CD4^+$ T cells expressing FoxP3 ($p<0.1$), while the $CD3^+CD8^+$ T cell population was slightly decreased ($p<0.09$) (Fig. 5b,d,e). No alterations on IFN-γ production by these cells was however observed (Fig. 5c,f). These results suggest that the reduction of tumor progression in the animals treated with RT combined with Ch/γ-PGA NPs was independent of T cell presence and activities at the primary tumor site.

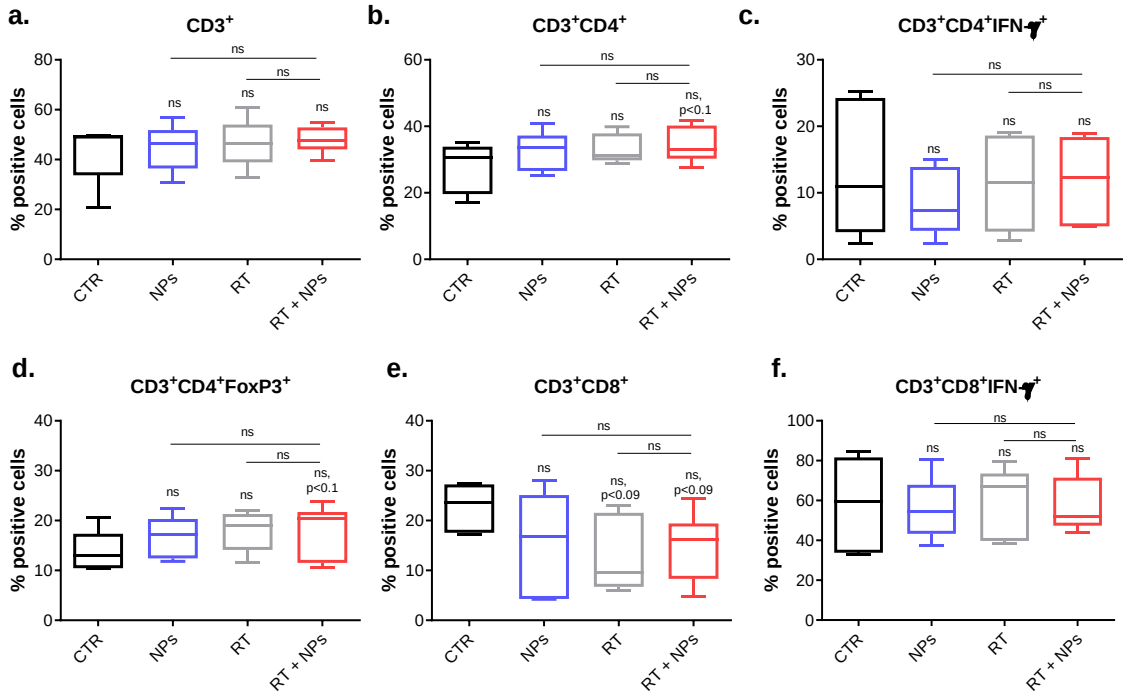

Figure 5. Ch/γ-PGA NPs plus RT slightly impact the accumulation of T cells at the primary tumor site. BALB/c mice injected with 4T1-Luc cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5 Gy) or treated with Ch/γ-PGA NPs (NPs) or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. Tumors were collected and processed for flow cytometry analysis. The T cells gate strategy was similar to the applied in the spleen analysis. $CD45^+$Live Dead $(LD)^-$ cells were gated on single cells (FSC-A vs SSC-H). $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were gated on $CD45^+LD^-$ cells. IFN-γ-producing T cells were gated on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. FoxP3-expressing cells were gated on $CD3^+CD4^+$ T cells. **a-f.** The percentage of positive cells was determined. Data is representative of at least 5 animals. Median is represented by the horizontal line inside the box plots. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^*p<0.05$ relative to non-treated control or single treatments (NPs or RT)).

3.6. Ch/γ-PGA NPs in combination with RT treatment decrease the levels of systemic immunosuppressive cytokines

The production of immune mediators, namely cytokines and chemokines, in the 4T1 model has been explored [31]. In order to characterize the systemic response to the different treatments, serum was analyzed through a multiplex analysis for a commercial panel of mouse cytokines and chemokines. We observed that 4T1 tumor inoculation enhanced the expression of cytokines with immunosuppressive and protumoral activities, namely, IL-3, IL-4, IL-6 and IL-10, in comparison to healthy animals (Fig. 6a). Importantly, no differences were observed between 4T1 tumor-induced and healthy animals regarding the expression of pro-inflammatory cytokines as, IL-12p40, IL-12p70, TNF-α and IFN-γ (Fig. 6b). Generally, NPs- and RT+NPs-treated animals presented lower immunosuppressive cytokines in the serum comparing to controls, while RT-treated animals had similar levels (Fig. c-f). Specifically, animals treated with the combination of RT with Ch/γ-PGA NPs experimented a reduction in 3.5-fold in IL-3 levels, 5.3-fold in IL-4 levels, 13-fold in IL-6 levels and 23-fold in IL-10 levels in comparison to RT-treated animals. This treatment also potentiated the NPs effect since decreased 2.1-fold IL-4 levels, 7-fold IL-6 levels and 2.3-fold IL-10 levels, in comparison to NPs-treated animals (Fig. 6c-f), which suggests NPs were the major driver in the reduction of immunosuppressive cytokines in the serum of animals treated with RT+NPs. Curiously, no major alterations in IL-12p40, IL-12p70, TNF-α and IFN-γ secretion were observed in treated animals (Fig. 6g-j). Overall, these results suggest that the combination of RT with NPs have a major impact on the reduction of immunosuppressive and protumoral cytokines in the serum, which could impact on the phenotype of immune cells recruited to the tumor site and, consequently on tumor progression.

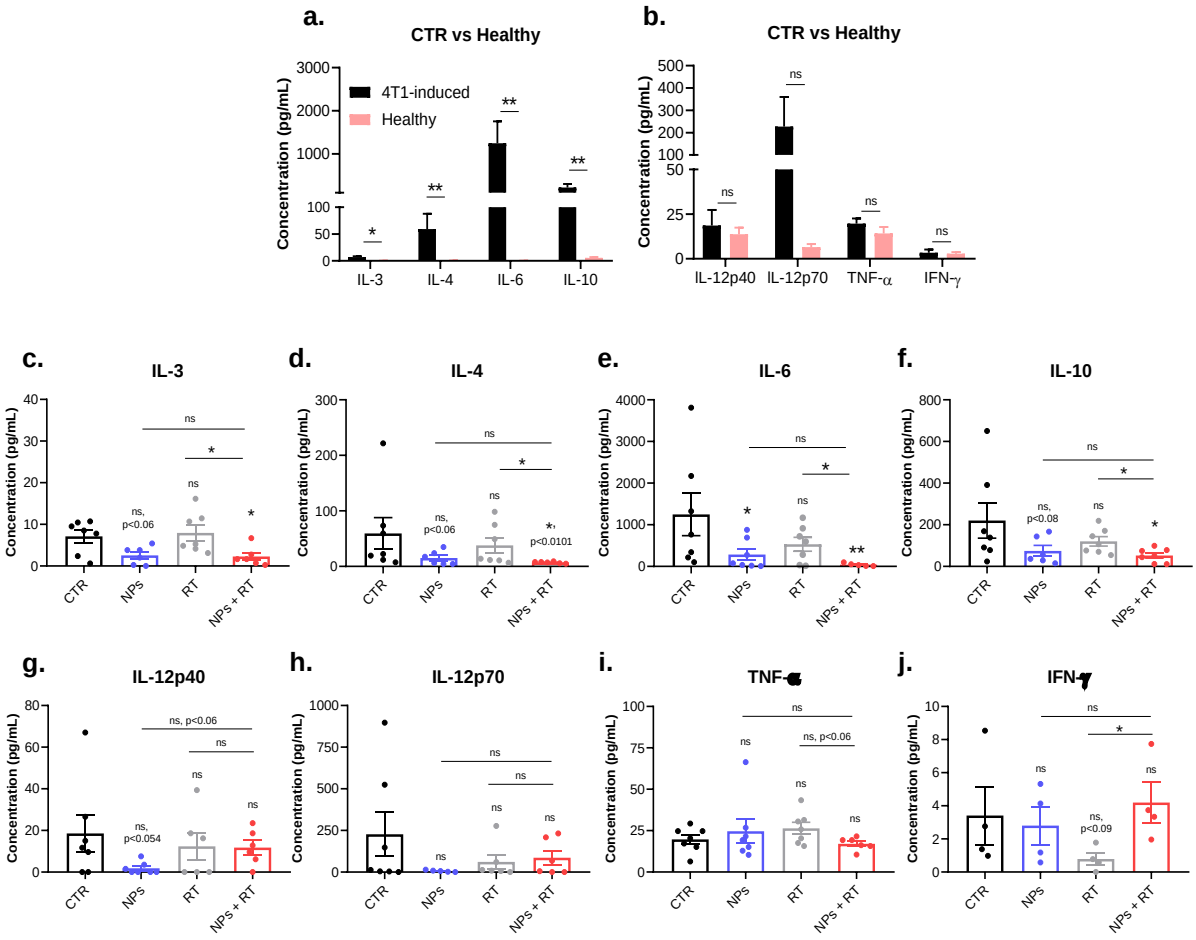

Figure 6. The decrease of immunosuppressive and pro-tumoral cytokines in the serum of animals treated with Ch/γ-PGA NPs plus RT is mediated by Ch/γ-PGA NPs treatment. BALB/c mice injected with 4T1-Luc cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5 Gy) or treated with Ch/γ-PGA NPs (NPs) or with RT combined with Ch/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. Blood of mice was collected at the end of experiment (day 28) and processed for serum collection. **a. to j.** Serum was analyzed through Multiplex immunobead assay technology. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 4 animals. **a. and b.** Serum from healthy animals was used as control. Comparisons were performed using the unpaired Mann-Whitney test (*p<0.05, **p<0.01 relative to non-treated animals). **c. to j.** All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test (*p<0.05, **p<0.01 relative to non-treated control or single treatments (NPs or RT)).

3.7. Ch/γ-PGA NPs in combination with RT treatment decrease lung metastasis burden

The 4T1 tumour model is highly malignant, being lungs and liver the main target organs [27]. Using whole-body fluorescence imaging, animals from different groups were imaged for 4 weeks but no differences regarding the bioluminescence signal in the lungs or in the liver were observed (data not shown). To better visualize the existence of putative metastasis, we collected the lungs and the livers at day 28 and proceeded to bioluminescence *ex vivo.* Curiously, we observed no significative differences in the bioluminescence levels quantified in the lungs of non-treated and treated animals (Fig. 7a). Similar results were observed in the liver (data not shown). Importantly, to complement this analysis, we performed haematoxylin and eosin staining in the lungs of treated and non-treated animals. Interestingly, control animals presented larger areas of the lungs metastasized than all treated animals (Fig. 7c). Importantly, NPs-, RT- and RT+NPs-treated animals presented smaller and lesser foci of metastases than non-treated animals. These experiments did not provide a quantification of lung metastasized area and further investigation is required. Additionally, we observed a decrease in the expression of molecules associated with lung metastasis in the serum of the animals treated with the combinatorial treatment, as CCL4, VEGF and GM-CSF (Fig. 7c,d and Fig. 3d), which can partially explain the reduced tumor burden in the lungs of these animals.

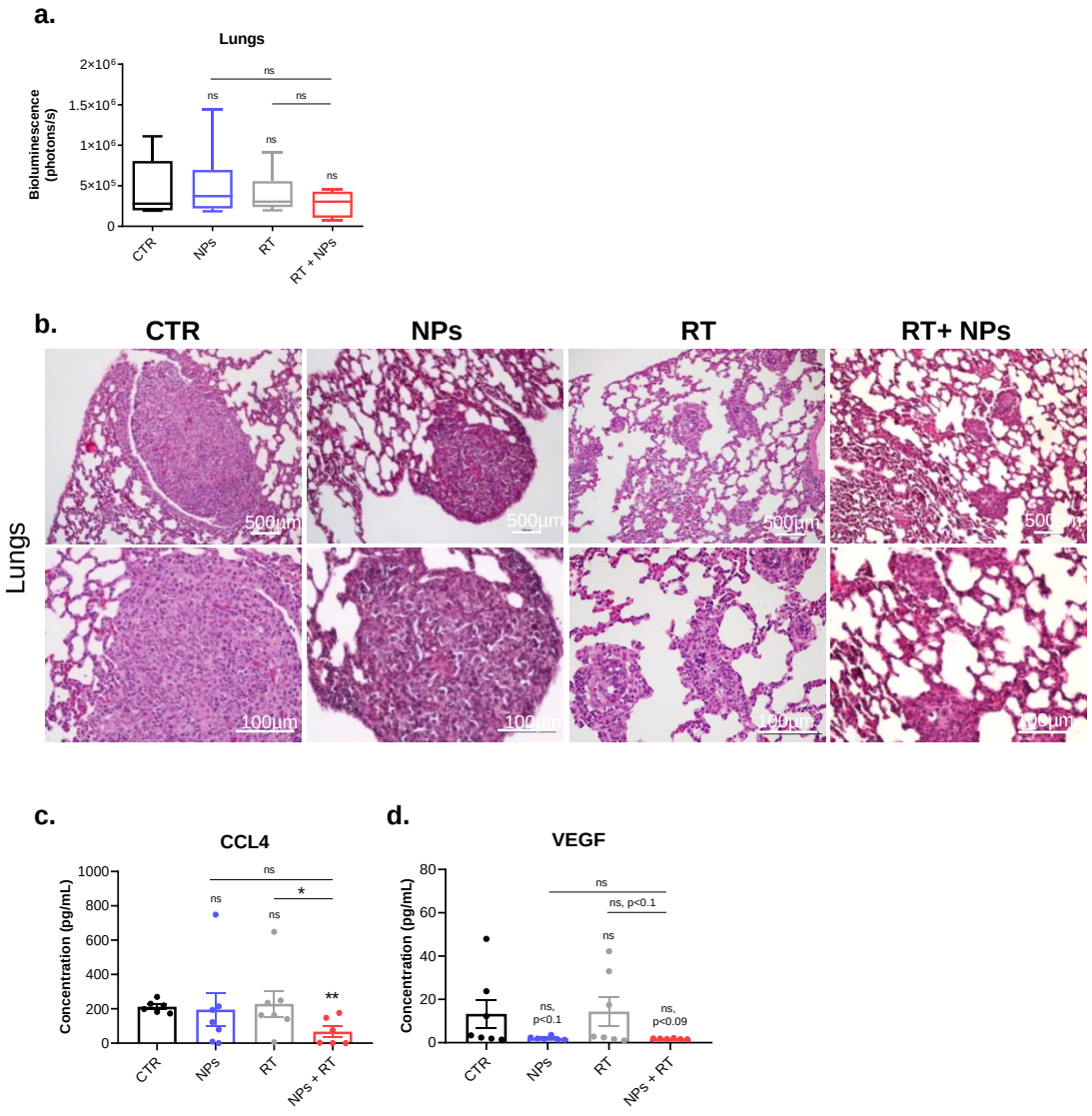

Figure 7. Ch/γ-PGA NP s plus RT decreases lung metastasis burden. BALB/c mice injected with 4T1-Luc cells on the mammary fat pad were submitted to radiotherapy (RT) (2x5 Gy) or treated with Ch/γ-PGA NPs (NPs) or with RT combined with C h/γ-PGA NPs (RT+NPs). Non-treated animals were used as control. Animals were bioluminescence imaged using a IVIS Lumina II every week to control tumor progression and metastasis formation. At day 28 (D28), animals were euthanized, and lungs were recovered for bioluminescence imaging *ex vivo*. **a.** Bioluminescence at the lungs was determined by drawing regions of interest (ROIs) and were expressed in total flux (photon/s), through IVIS software. Median is represented by the horizontal line inside the box plots. **b.** Lungs were processed for histological analysis and stained with hematoxylin and eosin, magnification 100x; scale bar: 500 µm; magnification 200x; scale bar: 100 µm. Data is representative of at least 7 animals. **c-d.** Serum was analyzed through multiplex immunobead assay technology. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 4 animals. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^{*}p<0.05$ and $^{**}p<0.01$ relative to non-treated control or single treatments (NPs or RT)).

4. DISCUSSION

The main obstacle to anticancer therapy success is the host tolerance established during tumor progression [32], which favors the acquisition of an immunosuppressive microenvironment, associated with the resistance to anticancer therapeutics [4, 33, 34]. Therefore, novel anticancer strategies should include immunomodulatory approaches to subvert the tolerance to the tumor. Specifically, RT induces immunogenic cell death, promoting the activation of the innate immune system and the recruitment of T cells to the tumor site. However, this response is only effective if immunosuppressive effects can be overcome. Several efforts have been done to target tumor microenvironment-mediated radioresistance mechanisms [4]. For example, RT followed by IGF-1R neutralization in orthotopic CRC models reduced the number of mice with metastases [35]. Additionally, preclinical studies have demonstrated the synergistic effects of RT combination with immunotherapies, namely anti-PD-1 or anti-CTL-4, which have demonstrated improved therapeutic response [36, 37], being currently under clinical trials [38].

In this preclinical study, we tested the combination of conventional RT and immunostimulatory Ch/γ-PGA NPs for treatment of metastatic breast cancer, by using a mouse model that closely mimics the traits of human disease, with metastases at lungs and liver. This therapeutic combination has never been studied before, and the current data provide the proof of concept that it can indeed elicit a significant antitumor immunity likely by impairing the immunosuppressive microenvironment. The statistically significant advantage obtained with RT and Ch/γ-PGA NPs resulted from the synergistic effects of the treatments. Whereas RT slightly decreased primary tumor burden, the Ch/γ-PGA NPs attenuated the systemic immunosuppression. Additionally, the increase in $CD4^+$ T cell response in the spleen of animals that experimented combinatorial treatment suggests that such strategy is likely to promote systemic antitumor immunity. In addition, the slight increase of effector T cells at the primary tumor could indicate that T cells are being recruited to the tumor site. Curiously, single treatments were not enough to promote T cell activation neither their infiltration into tumors, probably due to the immunosuppressive environment created by the established 4T1 tumors. In support of this possibility, it is known that primary 4T1 tumors are constituted by a high percentage of myeloid cells ($Gr1b^+$ $CD11b^+$), which generally increase with tumor progression [27, 39]. These cells can inhibit T cell activities [40] and promote lung metastasis formation [39]. We are currently investigating whether the expansion of $Gr1^+$ $CD11b^+$ myeloid cells during tumor progression is affected by treatment with RT+NPs. The reduction of IL-3, IL-6 and GM-CSF levels in the serum can suggest a decrease in $Gr1b^+$ $CD11b^+$ myeloid cells recruitment to the tumor site [41]. More importantly, the attenuation of leukemic reaction in the spleen of the RT + NPs-treated animals can be

an explanation for the better outcome of these animals, but further experiments are required.

The impairment of lung metastasis through the combination of RT with NPs treatment is partially mediated by a lesser immunosuppressive (IL-3, IL-4, IL-6 and IL-10) environment. In fact, others have shown the impact of these cytokines on the metastatic process. For example, RNAi-mediated silencing of IL-4Rα, a component of the IL4 receptor, was enough to reduce growth at metastatic sites in mammary tumor models. Similar results were obtained in IL4-deficient mice [42]. Others demonstrated that targeting IL-4 signaling sensitized breast cancer cells to anticancer therapy and strengthened immune responses by increasing $CD8^+$ T cells-expressing IFN-γ [43]. Furthermore, IL-6/JAK/STAT3 axis has been associated with tumor progression and metastasis [44] and a variant of IL-6 is associated with metastasis in breast cancer patients [45]. IL-10 levels in the serum has also been associated with poor prognosis in several types of cancer [46]. Additionally, the decrease in pro-angiogenic factors, as IL-3 and VEGF, could contribute for the reduction of metastasis observed in the animals treated with RT+NPs. For example, the targeting of IL-3 hampered tumor vessels formation [47], while VEGF inhibition has been associated with the suppression of breast cancer metastasis [48]. Importantly, CCL4 also was reduced in the serum of animals treated with RT in combination with NPs, and this chemokine was associated to breast cancer metastasis to bone [49]. Finally, the unveiling of $Gr1^+$ $CD11b^+$ myeloid cell dynamics in the lungs and liver can help us to understand the impact of RT + NPs on the metastatic process.

Previous studies using a single dose of 12 Gy, in the presence or absence of anti-CTLA-4, showed a delay in the growth of 4T1 irradiated tumor, although no statistical differences were reached. Additionally, animals treated with RT+anti-CTL-4 had an increased survival in comparison to single treated animals, which correlated with lung metastasis inhibition [36], suggesting that both treatments synergize to potentiate the antitumor response. In our study, we do not know yet how the distinct treatments are synergizing, but we suspect that Ch/γ-PGA NPs by activating Toll-like receptors 2 and 4, trigger the innate immunity and activate a pro-inflammatory immune response. Previous *in vitro* studies evidenced that Ch/γ-PGA NPs revert immunosuppressive macrophages (IL-10-treated) towards an immunostimulatory profile [24], which is in agreement with the decrease of immunosuppressive cytokines in the serum of NPs-treated animals. Surprisingly, the levels of pro-inflammatory cytokines (IL-12p40, IL-12p70, TNF-α, IFN-γ and IFN-γ-inducible chemokines) were not increased in the serum of NPs-treated animals, as we observed in *in vitro* upon macrophages or DCs stimulation with NPs. However, the ratio of pro-/anti-

inflammatory cytokines was increased, contributing to the delayed tumor progression and lung metastasis burden.

In summary, the present study demonstrated that is possible to achieve a systemic therapeutic immune response against a poorly immunogenic and spontaneously metastasizing mammary carcinoma by the combination of irradiation of primary tumor *in situ* with Ch/γ-PGA NPs administered locally. This technically simple and relatively low cost radioimmunotherapy has the advantage of Ch/γ-PGA NPs besides the immunomodulatory potential, to be used as carries for drugs, proteins, peptides, protecting them from degradation; to be combined with targeted antibodies or be delivered locally without pathological reaction.

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CHAPTER VI

Chitosan/poly(γ-glutamic acid) nanoparticles synergize with IFN-γ to induce antitumor immunity

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ABSTRACT

Interferon-γ (IFN-γ) has been identified as a key factor for antitumor immunity and response to immunotherapy. However, its use in the clinic remains restricted to chronic granulomatous disease and malignant osteopetrosis. In cancer, most of the clinical trials reported a limited potential of IFN-γ as single therapy. Currently, some clinical trials are evaluating its potential when combined with immunotherapies, despite preclinical evidences of their advantage are missing.

Here, we addressed the potential synergistic effects of chitosan (Ch)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) combined with IFN-γ in the immunocompetent 4T1 orthotopic breast tumor mouse model. We have previously reported Ch/γ-PGA NPs as a strategy to modulate immature or immunosuppressive antigen-presenting cells (APCs) to an immunostimulatory profile, leading to T cell activation and impairing APCs-mediated cancer cell invasion. Therefore, these immunomodulatory abilities of Ch/γ-PGA NPs point them as appealing adjuvants to IFN-γ-based therapies.

In this study, the impact of Ch/γ-PGA NPs used in combination with soluble IFN-γ administration on breast primary tumor burden, lung metastasis formation and immune cell profile was explored. Therefore, four experimental groups were considered: non-treated, treated with NPs, with IFN-γ or with the combination of both treatments (NPs+IFN-γ). While non-treated animals had progressive tumor growth and developed lung metastasis, NPs- and IFN-γ-treated animals significantly decreased primary tumor burden. Remarkably, when both treatments were combined, breast tumor growth was blocked. Still this effect was not sustained after treatment administration blockade. Notably, NPs+IFN-γ treatment potentiated the NPs effect in 54% and IFN-γ in 38%. Additionally, animals from the NPs+IFN-γ group presented lower bioluminescence in the lungs than other groups, which indicate lower metastatic burden. Systemically, the immunosuppression was slightly attenuated, and the percentage of myeloid cells was reduced in the combinatorial treatment. Overall, these results suggest that Ch/γ-PGA NPs potentiate and synergize with IFN-γ to reduce tumor progression, opening new perspectives to be used in anticancer strategies.

1. INTRODUCTION

Macrophages are innate immune cells whose activities are triggered in response to microenvironment stimuli. They display remarkable functional and phenotypical plasticity and can change their physiology in response to different signals, being involved in host defense, immune regulation and tissue repair [1]. In cancer context, macrophages have been assumed as essential maestros of the tumorigenesis process [2].

Tumor-associated macrophages (TAMs) are a major component of the tumor inflammatory infiltrate and are involved in various aspects of immunity, influencing the tumor outcome. Within the tumors, they sense the tumor microenvironment and can reversibly adjust their function in response to environmental cues, including signals derived tumor and stromal cells, such as immune cells, fibroblasts and adipocytes, as well as from extracellular matrix [3] and the hypoxic environment [4]. Depending on their activation status, macrophages can exert dual roles on tumorigenesis by either enhancing the antitumor immunity or by creating an immunosuppressive environment which consequently dampens the cytotoxic actions of immune cells. In fact, in most of solid cancers they exert immunosuppressive activities and increased infiltration of TAMs has been associated with poor patient prognosis [5], highlighting their potential as targets for anticancer therapies.

Macrophage functional classification comprises a wide spectrum, but it is conventionally accepted that macrophages can differentiate into two extreme phenotypes: the “classically activated” M1 macrophages or the “alternatively activated” M2 macrophages [6]. For M1 macrophage polarization, this typically involves interferon-γ (IFN-γ), pathogen products, as lipopolysaccharides (LPS), or tumor-necrosis factor-α (TNF-α) stimulation. They are associated with pro-inflammatory, microbicidal and tumoricidal activities, stimulation of T cell immunity [7], high antigen-presentation abilities and enhanced secretion of interleukin (IL)-12, IL-23, IL-6, IL-1β, reactive oxygen species and low levels of IL-10. In opposite, M2-like macrophages may be induced by IL-4, IL-13, IL-10, transforming-growth factor (TGF-β), immune complexes and glucocorticoid factors. They generally secrete high levels of IL-10 and TGF-β and low levels of IL-12, TNF-α and IL-6, have the ability to impair T cell responses [8] and are involved in tissue repair, proteolysis, angiogenesis and tumor progression [9]. TAMs are frequently associated to M2-like profile although they can comprise different phenotypes depending on tumor stage [10, 11]. Recently, our group showed that M1 and M2-like macrophages have different abilities to promote *in vitro* cancer cell invasion and migration as a result of different proteolytic activities [12]. Considering macrophages functional plasticity and their detrimental role in cancer progression, several

therapeutic strategies have been designed for TAMs depletion [13, 14] and most recently, several studies revealed the potential of reprograming TAMs towards an antitumoral profile [15-17].

Previous studies have shown that the combination of different stimuli for macrophage activation can potentiate a stronger M1 phenotype, namely through the combination of IFN-γ with LPS [18, 19]. IFN-γ was identified 36 years ago as the major player of macrophage tumoricidal action [20]. Later studies suggested that IFN-γ may not be enough and that a second signal from the microenvironment might be required [21, 22]. Besides its tumoricidal activity, IFN-γ is well known by its immunomodulatory properties, being crucial for an effective antitumor response [23]. Recent works demonstrated that IFN-γ reverted the immunosuppressive and protumoral phenotype of ovarian TAMs [24] and human IL-10-educated macrophages [25] towards a M1-like profile, thus appearing as an interesting molecule for immunomodulatory therapeutic strategies. In fact, IFN-γ priming has been shown to enhance macrophage activation through TLR signaling upregulation or by desensitizing macrophages to the immunoregulatory effects of adenosine and IL-10 [26-28], while the additional signal provided by Toll-like receptor (TLR) agonists, as LPS or other pathogen-associated molecular pattern molecules, triggers a maximal cytotoxic macrophage response [29]. TLR agonists have been explored for their potential use in anticancer therapies, either as vaccine adjuvants or immunomodulators, in order to reprogram immunologically “cold” into “hot” tumors [30]. Several TLR agonists were described to activate macrophages, inducing a pro-inflammatory response [29, 31, 32] and the antitumor responses associated to TLR agonists are largely attributed to their capacity to stimulate antigen-presenting cells (APCs), namely macrophages and dendritic cells which in turn, activate tumor-specific T cell responses [33-35]. On their turn, biomaterials with immunostimulatory properties have been studied for anticancer therapy. Poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) have been described to induce potent innate and adaptive immune responses through TLR4 and MyD88 signaling pathways [36], while chitosan (Ch) was shown to activate the NLRP3 inflammasome, leading to robust IL-1β responses by a phagocytosis-dependent mechanism [37]. We have recently reported that Ch/γ-PGA NPs reprogram immunosuppressive IL-10-treated macrophages and immature dendritic cells towards an immunostimulatory profile. These phenotypic alterations differentially potentiated the proliferation and activation of T cells and hampered their ability to induce cancer cell invasion [38]. This prompted us to investigate the potential synergistic effects of these immunomodulatory Ch/γ-PGA NPs with tumoricidal IFN-γ in inducing a potent and sustainable antitumor response. Thus, the aims of the present work were to

evaluate the ability of Ch/γ-PGA NPs to synergize with IFN-γ to (1) reprogram the immune response, (2) impair primary tumor progression, and (3) inhibit lung metastasis. This report highlights the potential of Ch/γ-PGA NPs to synergize with IFN-γ to induce an antitumor response, characterized by a decrease in myeloid cells and an increase on CD4 T cell response, resulting in a better control of breast tumor progression.

2. MATERIALS AND METHODS

2.1. Ch/γ-PGA NPs preparation and characterization

Ch/γ-PGA NPs were prepared by co-acervation method as previously described [38]. Briefly, Ch (France-Chitine) with the degree of acetylation (DA) of 10.4±1.6%, determined by Fourier transform infrared spectrometry using KBr pellets (FTIR-KBr), and molecular weight (MW) of 324±27 kDa, determined by size-exclusion chromatography, was used. γ-PGA, with 10-50 kDa and 99.5% purity, was produced from *Bacillus subtilis* cultures as described by Pereira *et al* [39]. Ch/γ-PGA NPs were prepared at a molar ratio of 1:1.5 (mol Ch:mol γ-PGA). γ-PGA solution (0.2 mg/mL in 0.05 M Tris-HCl with 0.15 M NaCl buffer solution) was dropped to a Ch solution (0.2 mg/mL in 0.2 M AcOH), using a 1 mL syringe copulated to a syringe pump (KD Scientific Inc., Holliston, MA), at constant speed (3.6 µL/s), under high stirring and at room temperature (RT). All solutions had the pH adjusted to 5.0. After initial preparation, Ch/γ-PGA NPs were concentrated 10 times by centrifugation (13000 rpm, 30 min 4ºC) followed by resuspension in one tenth of the initial volume of buffer solution. Ch/γ-PGA NPs size, polydispersion index and zeta potential was evaluated by dynamic light scattering (DLS) in a ZetaSizer Nano Zs (Malvern Instruments), equipped with a He-Ne laser (λ= 633 nm). Each NPs batch produced was tested and 3 measurements per sample were acquired.

2.2. Ethics Statement

Human samples were obtained in agreement with the principles of the Declaration of Helsinki. Monocytes were isolated from surplus buffy coats from healthy blood donors. These were kindly provided by the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ) from Porto, Portugal. This is covered by the ethical approval of the service, under which blood donors give informed written consent for the byproducts of their blood collections to be used for research purposes (Protocol reference 260/11). All animal experiments were performed following strictly the recommendations of the European Union

Directive 2010/63/EU and previously approved by the local ethics committee of Ghent University Hospital (ECD 17/124). Mice were euthanized by cervical dislocation with efforts to minimize suffering.

2.3. Human monocyte isolation and macrophage differentiation

Human monocytes were isolated from buffy coats from healthy blood donors, as previously optimized in our lab. Briefly, peripheral blood mononuclear cells (PBMC) were collected from centrifuged buffy coats (30 min, 1200xg, RT, without brake) and incubated with RosetteSep human monocyte enrichment kit (StemCell Technologies), according to manufacturer's instructions. The mixture was diluted 1:1 with PBS supplemented with 2% FBS (heat inactivated fetal bovine serum, Biowest), layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged as before. The enriched monocyte layer was collected and washed with PBS. Following this procedure, over 80% of isolated cells were found to be CD14-positive. For monocyte-macrophage differentiation, $1x10^5$ monocytes/3,8cm^2 (24-wells plate) were cultured for 10 days in complete RPMI1640 medium, supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin, in absence of M-CSF or other exogenous factors. For M1-like profile, macrophages were incubated with 10 ng/mL LPS (Sigma-Aldrich) or with 100 ng/mL IFN-γ (Immunotools) while for M2-like profile macrophages were stimulated with IL-10 (ImmunotoTools), for an additional 72 h. Ch/γ-PGA NPs (0.7 mg/mL) were added to IL-10-stimulated macrophages at 1% (v/v) after 4 h of IL-10 stimulation, for another 72 h.

2.4. Macrophage profile by flow cytometry

For cell surface receptor expression analysis, macrophages were incubated with accutase (eBioscience) at 37ºC during 30 min and harvested by gently scrapping. Cells were washed and resuspended in FACS buffer (PBS, 2% FBS (Biowest), 0.01% sodium azide) containing appropriate conjugated antibodies, and stained in the dark for 45 min at 4ºC. Macrophages were immunostained with the following antibodies: anti-human CD14-APC (clone MEM-18), CD86-FITC (clone BU63), HLA-DR-FITC (clone MEM-12) (Immunotools) and CD163-PE (clone GHI/61) (R&D Systems). To define background staining isotype matched antibodies were used as negative controls. After additional washing steps, cells were acquired on a FACS Canto Flow Cytometer (BD Biosciences) with BD FACSDiva software. Results were analyzed using FlowJo software version 10 (TreeStar, Inc.) and analyzed as above. Median fluorescence intensity was calculated by subtracting the respective isotype control intensity.

2.5. Macrophage profile by ELISA

Macrophages supernatants were collected after 72 h of stimulation. IL-12/IL-23p40, IL-6, IL-1β and IL-10 secreted levels were determined by enzyme-linked immunosorbent assay (ELISA) through Human Legend Max™ Kits (Biolegend), according to the manufacturer's instructions.

2.6. Cell lines

The 4T1 cells expressing luciferase (4T1-Luc), a mouse triple negative breast cancer cell line, was obtained from Sibtech (Brookfield, CT, USA). Tumor cells were defrosted and maintained/expanded in Dulbecco's Modified Eagle's Medium (Gibco; Life Technologies), supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco; Life Technologies) and 1% (v/v) penicillin/streptomycin (Sigma) (cDMEM) at 37°C and 5% CO_2. All cultures were regularly tested for Mycoplasma by using MycoAlert Plus Kit (Lonza, Basel, Switzerland).

2.7. IncuCyte ZOOM monitored assays

Real-time monitoring of cell confluency was done using IncuCyte ZOOM System (Essen Bioscience, Hertfordshire, UK) according to the manufacturer's guidelines. For cell confluency monitoring, cells were seeded in Corning 96-well plates (1,000 cells/well, 100 µL/well) and allowed to adhere overnight at 37ºC and 5% CO_2. Subsequently, cells were treated with conditioned medium collected from macrophage cultures. Microscopic images (4 images/well) were taken every 2 h for the indicated time. Images were analyzed, and cell confluency was deduced using IncuCyte software. Each condition was performed at least six times and each experiment was performed in triplicate.

2.8. Cell metabolic activity

The metabolic activity of macrophages was determined by resazurin reduction assay. Briefly, after 72 h of stimulation, cells were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich) for 4ºC at 37ºC and 5% CO_2. Fluorescence intensity was measured at 590 nm in a Synergy MX plate reader (BioTek), and values normalized to unstimulated cells.

2.9. Animal studies

Four-week-old female immunocompetent BALB/cByJ mice (Charles River Laboratories, l' Arbresle Cedex, France) were injected orthotopically in the mammary fat pad with $1x10^6$ 4T1-Luc cells, resuspended in 100 µl serum-free DMEM with Matrigel matrix (1:1) (Corning).

Tumor subcutaneous implantation was confirmed by bioluminescence imaging after 24h of tumor cell injection. Mice were intraperitoneally injected with 150 mg/kg body weight D-luciferin (Caliper Life Sciences), 20 minutes (min) before bioluminescence imaging, which was carried out by using an IVIS Lumina II (Caliper Life Sciences). Bioluminescent imaging was initiated by a cooled CCD camera through IVIS monitorization with a 15-cm field of view, binning factor of 8, 1/f stop and open filter. Exposure times were set automatically, according to luciferase signaling activity. Regions of interest (ROIs) were drawn for primary tumor and metastatic sites and calculated through the IVIS software, expressed in total flux (photon/s). After seven days of tumor cell inoculation, when the tumors reach around 50 mm^3, animals were treated subcutaneously near the tumor, 6 times for 2 weeks with Ch/γ-PGA NPs (NPs) (0.7 mg/mL), IFN-γ (13.6 µg/mL) (IFN-γ) or with or both (NPs + IFN-γ). Tumor volume was measured using a caliper and calculated as (length × width × width)/2 (mm^3) and tumor progression was followed by bioluminescence imaging every week. Animals were sacrificed at day 33.

2.10. Spleen digestion and stimulation

Spleens were collected 4 weeks after tumor cell inoculation and were mechanically digested. Erythrocytes were lysed with red blood cell lysis buffer (Biolegend) for 3 min. Spleen cells for myeloid staining were stimulated with 1 µg/mL of LPS (Sigma) and 10 ng/mL of recombinant mouse interferon-gamma (Peprotech) for 4 hours (h) at 37°C. For lymphoid staining, tumor cells were stimulated with Phorbol 12-myristate (PMA) (200 ng/mL, Sigma), ionomycin (1 µg/mL, Sigma) and Brefeldin A (BFA) (10 µg/µL, Sigma), for 4 h at 37°C.

2.11. Flow Cytometry analysis

After stimulation, cells were washed in FACS buffer (PBS, 2% FBS, 0.01% sodium azide) and pre-treated with Fc block (anti-mouse CD16/CD32, Biolegend) for 10 min to minimize a non-specific antibody binding. Then, cells were extracellular stained with specific conjugated antibodies, in the dark, for 45 min at 4ºC. The following antibodies were used: CD3-PerCPcy5.5 (clone145-2C11, eBioscience), CD4-BV421 (clone RM4-5, Ebioscience), CD8 (clone 53-6.7, Biolegend), CD11b-Alexa 700 (clone M1/70, Biolegend), CD11c-FITC (clone N418, Biolegend), CD45-BV510 (clone 30-F11, Biolegend), CD45-APC (clone 30-F11, Biolegend), CD206-PerCPcy5.5 (clone C068C2, Biolegend), Ly6C-BV605 (clone HK1.4, Biolegend), Ly6G-BV421 (clone 1A8, Biolegend), F4/80-PEcy7 (clone BM8, Ebioscience) and MHC II-APCcy7 (clone M5/114.15.2, Ebioscience), PD-1-FITC (clone

J43, Ebioscience) and PD-L1-PEDazzle (clone 29E.2A3, Biolegend). The Aqua Zombie-BV510 and Live Dead-APC-Cy7 were used to confirm cell viability. Cells were washed and fixed with 2% of paraformaldehyde overnight and then permeabilized with Permeabilization buffer (Invitrogen), in the dark, for 20 min at RT. Then, cells were intracellular stained for intracellular antigens for 30 min at RT. The following antibodies were used: IFN-γ-BV605 (clone XMg1.2, Biolegend), TNF-α-PE (clone MP6-XT22, Ebioscience) and FoxP3-APC (clone FJK-16S, Ebioscience). Isotype-matched antibodies were used as negative controls. After additional washes, cells were acquired on a BD LSRFortessa Flow Cytometer (BD Biosciences) and analyzed as above.

2.12. Serum collection and analysis

Blood was collected by cardiac puncture for non-coated eppendorfs. Then, blood samples were centrifuged at 2500 rpm, for 30 min at RT. The serum fraction collected was centrifuged to remove erythrocytes at 1200 rpm, 5 min, at 4ºC and stored at -80ºC until use. The samples were analyzed for cytokines and chemokines using a mouse cytokine array/chemokine array 31-multiplex (MD31) through Eve Technologies services.

2.13. Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software version 7 (GraphPad-v7). Due to the limited number of donors included in test groups ($n \leq 7$), a non-Gaussian distribution of the data was considered for the statistical analysis. Therefore, the non-parametric Kruskal Wallis test was used for non-paired comparisons between groups. Statistical significance was achieved when *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3. RESULTS

3.1. Ch/γ-PGA NPs plus IFN-γ treatment prevent IL-10-induced polarization and synergizes to induce an immunostimulatory macrophage profile

The activation profile of macrophages present at the tumor microenvironment influences tumor and stromal cells activities. As their immunosuppressive functions are frequently associated with tumor progression, the reprogramming of their profile towards a pro-inflammatory phenotype appears as a promising therapeutic strategy. Thus, macrophages treated with IL-10 for M2 polarization, were posteriorly treated with Ch/γ-PGA NPs (IL-10+NPs), IFN-γ (IL-10+IFN-γ) or with NPs plus IFN-γ (IL-10+NPs+IFN-γ) to evaluate whether the combination of both treatments further potentiate the immunostimulatory ability of the single treatments. The cellular surface expression of CD14, a lineage marker, the co-stimulatory CD86 and the scavenger receptor CD163, M1- and M2-like markers, respectively, was evaluated by flow cytometry. Naïve macrophages and LPS-treated were also analyzed for control purposes (data not shown). Our results evidenced that NPs stimulation did not alter neither the expression of CD14 nor of CD86 while decreased CD163 MFI and the percentage of $CD163^+$ cells (Fig. 1a-f). IFN-γ stimulation led to a CD14 expression slightly increase, without reaching statistical significance (Fig. 1a,b). IFN-γ stimulation significantly increased CD86 expression but the percentage of $CD86^+$ cells was not affected (Fig. 1c,d) whereas the expression of CD163 was not altered (Fig. 1e,f). The combination of NPs with IFN-γ did not significantly impact on CD14 expression (Fig. 1a,b). Importantly, the CD86 MFI and the percentage of $CD86^+$ cells was further increased comparing to NPs or IFN-γ single treatments ($p<0.1$) (Fig. 1c,d) while the percentage of $CD163^+$ cells decreased in comparison to IFN-γ or IL-10 treatments (Fig. 1e,f). Thus, these results suggest that the combination of NPs with IFN-γ have a synergistic effect, retaining the impact of individual stimuli and further potentiating the increase of the co-stimulatory CD86 expression. To further characterize the impact of this combinatorial treatment on macrophages, we profiled the cytokine production of these cells. NPs stimulation induced a significant production of pro-inflammatory cytokines, namely IL-12/IL-23p40, IL-6 and IL-1β, suggesting their ability to counteract IL-10 immunosuppressive effect. Interestingly, IFN-γ-treated macrophages did not increase the production of the pro-inflammatory cytokines neither decreased the levels of the anti-inflammatory one. Importantly, the IFN-γ+NPs combinatorial treatment significantly enhanced the secretion of pro-inflammatory cytokines, in comparison to IL-10 or IL-10+IFN-γ-treated macrophages, but no differences were observed to NPs-treated macrophages, suggesting the major role of Ch/γ-PGA NPs in the induction of the secretion of these cytokines (Fig. 1g-j).

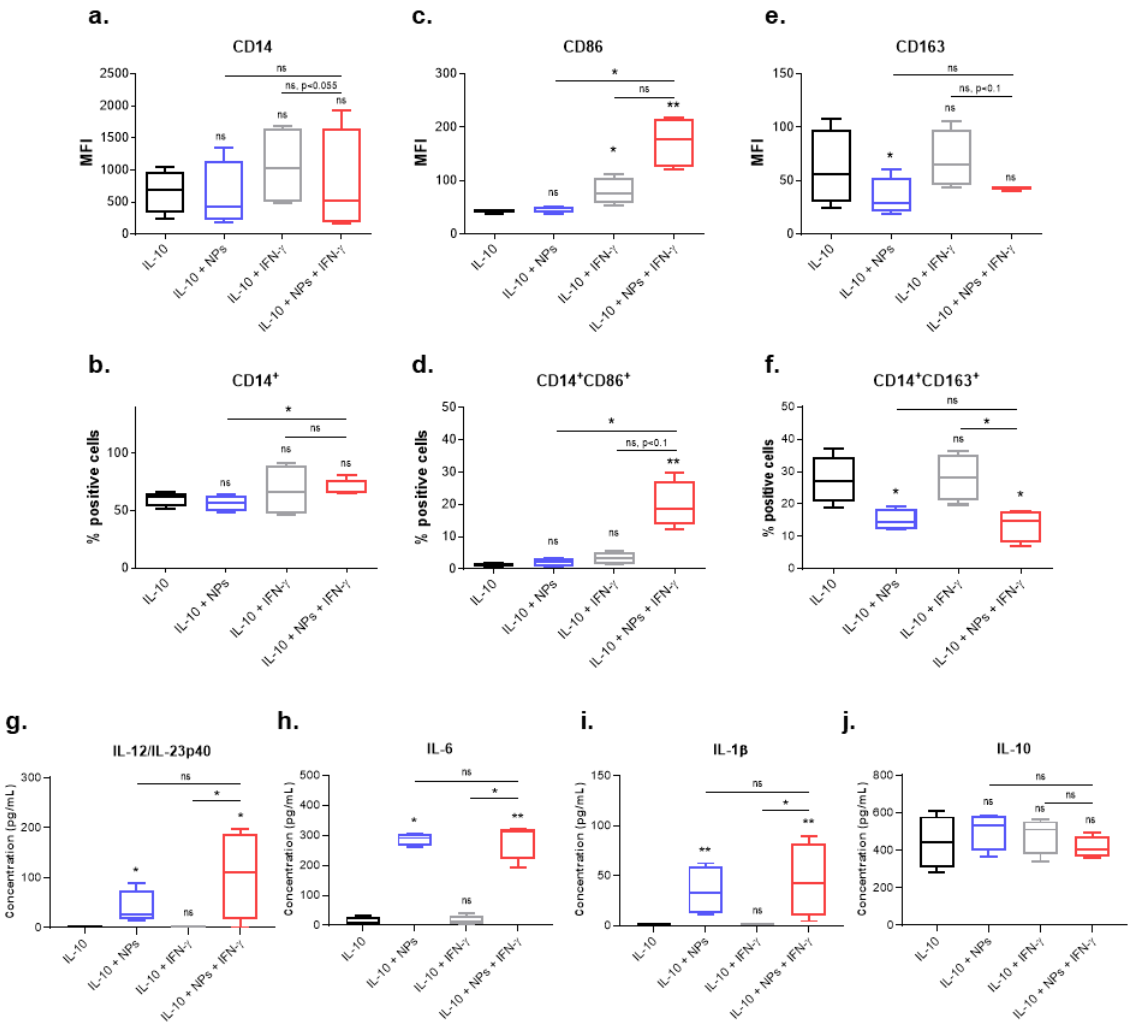

Figure 1. Ch/γ-PGA NPs and IFN-γ synergize to induce an immunostimulatory macrophage profile. Macrophages were stimulated with IL-10 during 72 h in order to polarize to M2-like macrophage profile. Simultaneously, IL-10-stimulated macrophages were treated with Ch/γ-PGA NPs (IL-10+NPs), IFN-γ (IL-10+IFN-γ) or with the combination of both (IL-10+NPs+IFN-γ) for 72 h. Cells were stained for the monocyte/macrophage lineage marker (CD14), the co-stimulatory M1 marker (CD86) and the scavenger receptor M2 marker (CD163), and were analyzed for flow cytometry. **a-c.** The MFI of CD14, CD86 and CD163 was calculated by subtraction of the fluorescence intensity of the respective isotype control. **d-f.** Percentage of positive cells was determined. **g-j.** Macrophage conditioned medium was collected and levels of pro-inflammatory cytokines (IL-12/IL-23p40, IL-6, IL-1β) and of the anti-inflammatory IL-10 were determined by ELISA. Data is representative of at least 4 donors. All comparisons were performed using the Friedman test followed by Dunn's multiple comparison test (*p<0.05; **p<0.001 relative to control or single treatments (NPs or IFN-γ)). Median is represented by the horizontal line inside the box plots.

3.2 IFN-γ seems to render a tumoricidal activity of macrophages treated with Ch/γ-PGA NPs combined with IFN-γ

To evaluate the functional impact of the combinatorial treatment on macrophages tumoricidal activity, we stimulated colorectal cancer RKO cells with conditioned medium (CM) from macrophages and followed, in real-time, cell proliferation using the IncuCyte ZOOM System. Our results evidence that the CM from IL-10-treated macrophages did not impact on the proliferation of RKO cells, similarly to what was observed with CM from naïve macrophages (CM Mac) or with just medium (control, CTR) (Fig. 2a-c). On its turn, CM from IL-10-treated macrophages with NPs induced a slight decrease in RKO proliferation, while the CM from IL-10-macrophages treated with IFN-γ or with NPs plus IFN-γ abrogated the RKO cell proliferation, after 24 h of treatment, probably by inducing cell death (Fig. 2a). This effect was dependent on soluble factors produced by macrophages after IFN-γ stimulation, since RKO cells stimulated directly with IFN-γ proliferated in a similar way compared to CTR (Fig. 2c). Additionally, the abrogation of RKO cell proliferation was also observed using CM from LPS-treated macrophages (Fig. 2b), which indicates that the acquisition of the M1-like profile is associated with tumoricidal activity, as previously reported [29]. Curiously, the pro-inflammatory response of macrophages induced by NPs did not impact RKO cell proliferation, which reinforces the combination of NPs with IFN-γ to potentiate the immunostimulatory and potential tumoricidal properties in a future anticancer therapeutic strategy.

In addition, RKO treated with CM from IL-10-treated macrophages remained viable, while apoptotic signs, as cell shrinkage and loss of membrane integrity, were observed in RKO cells submitted to CM from IL-10-macrophages treated NPs plus IFN-γ (Fig. 2d) or treated just single IFN-γ (data not shown). Furthermore, CM from IL-10 and IL-10+NPs did not affect RKO metabolic activity, whereas CM from IL-10-macrophages treated with IFN-γ or with NPs plus IFN-γ decreased 8-fold (Fig. 2e). CM from macrophages treated with LPS or with IFN-γ also decreased cancer cell metabolic activity (Fig. 2f), in agreement with previous proliferation results. In contrast, the direct treatment of RKO cells with stimuli IL-10, LPS, IFN-γ or NPs had a small impact in their metabolic activity (Fig. 2g). Despite further studies are required, the combination of NPs with IFN-γ seems to render a macrophage tumoricidal phenotype in an IFN-γ-dependent way.

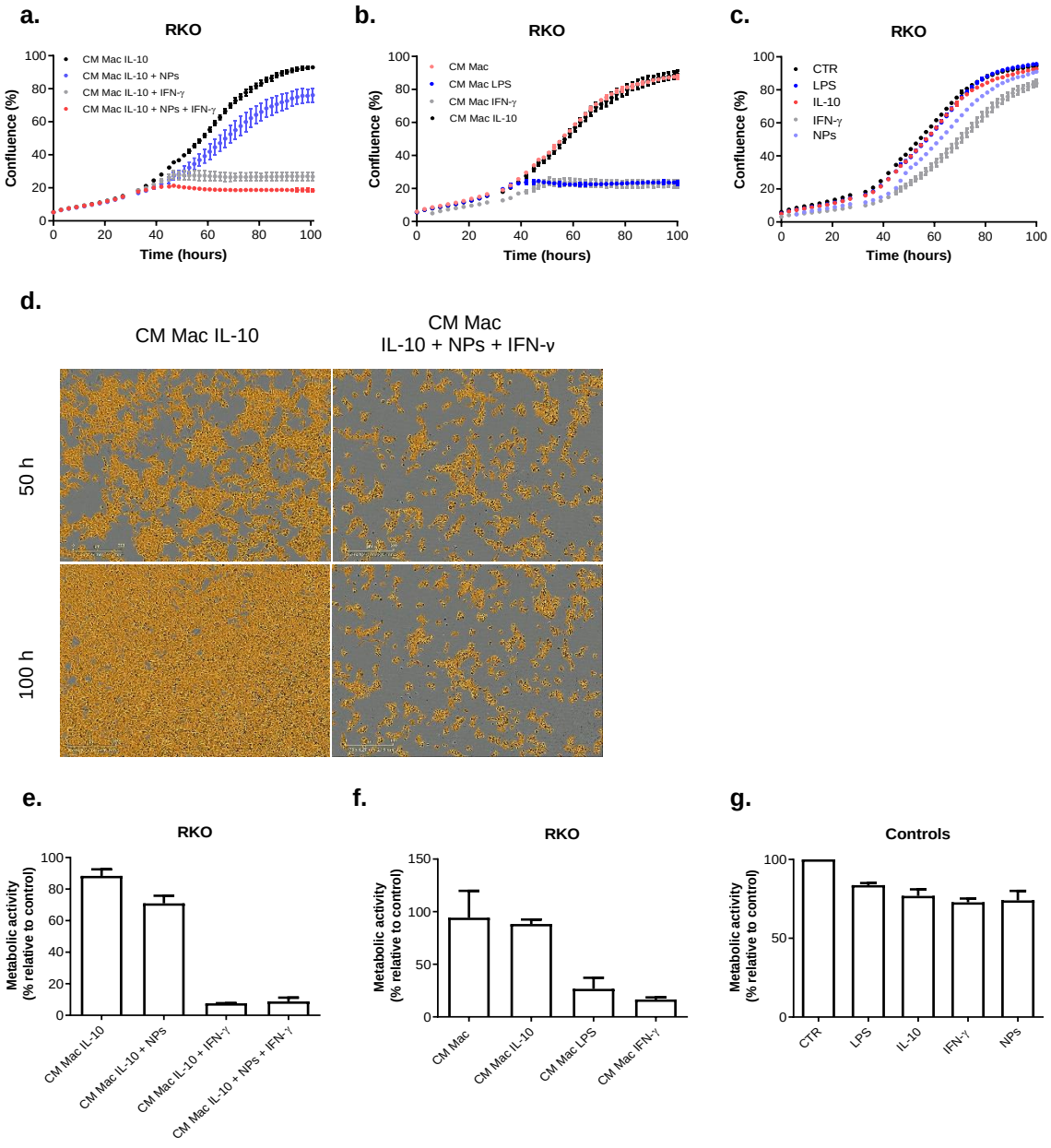

Figure 2. Ch/γ-PGA NPs plus IFN-γ seems render a tumoricidal activity in an IFN-γ-dependent way. Macrophages were stimulated with LPS, IFN-γ or IL-10 during 72 h in order to polarize to M1- or M2-like macrophage profile. Simultaneously, IL-10-stimulated macrophages were treated with Ch/γ-PGA NPs (IL-10+NPs), IFN-γ (IL-10+IFN-γ) or with the combination of both (IL-10+NPs+IFN-γ) for 72 h. Culture supernatants, designated as conditioned media, were collected and used for RKO cells stimulation. RKO cells were also stimulated directly with LPS, IL-10, IFN-γ or NPs as controls. **a-d.** Real-time analysis of cell confluency of treated RKO cells using IncuCyte technology. Microscopic pictures were taken every 2 h and confluency was determined using IncuCyte software. In **a-d.** a representative example of three biological donors is represented. Mean ± SEM of four technical replicates is shown. **e-g.** Metabolic activity was evaluated by resazurin assay and values were normalized to control (RKO cells treated with medium). Bars represent mean values with error bars representing ± SEM. Data is representative of two donors.

3.3. Ch/γ-PGA NPs plus IFN-γ treatment does not impact mice body weight

Following the promising *in vitro results*, we set to explore whether NPs plus IFN-γ modulate the immune response and tumor progression of solid tumors *in vivo.* For that, we used a murine mammary 4T1 cells, well described in the literature and recognized by their poor immunogenicity and high malignancy in a BALB/c syngeneic orthotopic model [40]. One week after luciferase-expressing breast cancer cells inoculation, NPs, IFN-γ or NPs plus IFN-γ were administrated subcutaneously, six times along two weeks, into mice bearing orthotopic 4T1 tumors, and whole-body bioluminescence imaging was performed, at different time points, to follow tumor progression. Therefore, non-treated animals (CTR) were used as experimental controls, and all animals were sacrificed for analysis at day 33 (Fig. 3a). The animal body weight was measured every week and no alterations were significantly observed, suggesting that the distinct treatments did not impact on animal condition (Fig. 3b). At the end of the experiment, day 33, liver and kidneys were harvested and stained with hematoxylin and eosin to evaluate the impact of possible accumulation of Ch/γ-PGA NPs in these organs, being these effects still under current analysis.

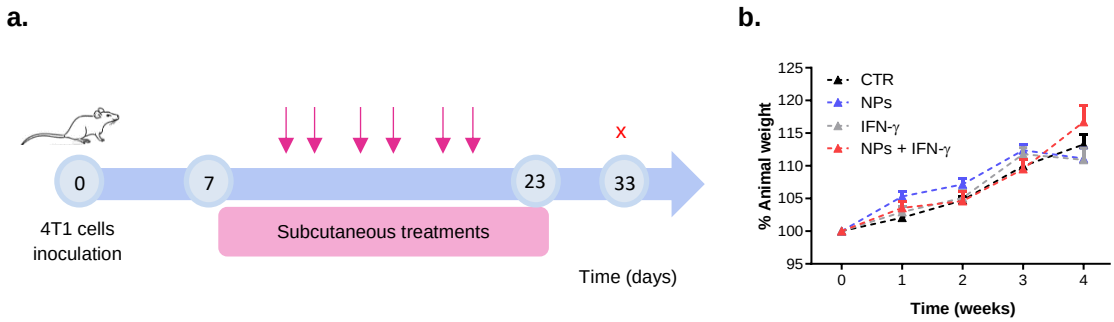

Figure 3. Ch/γ-PGA NPs plus IFN-γ treatment does not impact on animal body weight. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated with Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+IFN-γ). Non-treated animals but injected with 4T1 cells were used as control. **a.** Experimental timeline. Animals were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs, IFN-γ or Ch/γ-PGA NPs+IFN-γ and left then 10 days without treatments. Animals were sacrificed at day 33. **b.** Animal body weight was evaluated every week for 5 weeks. Time 0 corresponds to the week before tumor cells inoculation. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test relative to non-treated control or single treatments (NPs or IFN-γ). No significant differences were found.

3.4. Ch/γ-PGA NPs plus IFN-γ treatment further decreases 4T1 primary tumor progression

IFN-γ is a cytokine currently used for chronic granulomatous disease and severe malignant osteopetrosis treatment, while in cancer its use is currently limited to immunotherapeutic clinical trials and cell stimulation in cell-based therapies. Although its controversial role in tumor immune response, its role on prognosis and therapeutic response is well accepted [41, 42]. Thus, we asked if the combination of NPs plus IFN-γ decreased breast primary tumor progression. Therefore, tumor growth was monitored through tumor volume measurements and bioluminescence imaging. Importantly, we observed that tumors from non-treated animals grew progressively while treated animals presented significant alterations in tumor progression (Fig. 4a). After 2 weeks of treatment (day 23), animals treated with NPs or IFN-γ presented a significant delay in tumor progression (286.5 ± 41.6 % or 262.0 ± 43.4 %, respectively) in comparison to control animals (514.7 ± 61.2 %). Interestingly, the combination of NPs with IFN-γ significantly blocked tumor progression (107.8 ± 24.5 %) (Fig. 4b). After the animals were left without treatments for 10 days, we verified that the effect of NPs plus IFN-γ was not sustained over time and the animals presented bigger tumors in comparison to the day 23 (352.2 ± 101.9 %) (Fig. 4c). Still, the combinatorial treatment potentiated the effect of NPs in 54 % and of IFN-γ in 38 % (Fig. 4c). These results were corroborated by bioluminescence imaging of animals, where the tumors treated with the NPs plus IFN-γ presented lower bioluminescence than control groups which indicates lower tumor burden (Fig. 4d). The quantification of bioluminescence, through total photon flux (photons/second, p/s), at the tumor region confirmed that NPs or IFN-γ single treatments, slightly impacted on tumor burden ($4.2x10^8 \pm 8.5x10^7$ p/s or $4.9x10^8 \pm 7.3x10^7$ p/s, respectively) in comparison to control animals ($1.4x10^9 \pm 5.1x10^8$ p/s), while the combination of NPs with IFN-γ significantly reduced tumor burden ($2.5x10^8 \pm 1.8x10^8$ p/s) (Fig. 4e). Overall, these results suggest that the combinatorial treatment better controls primary tumor growth and acts synergistically to single treatments.

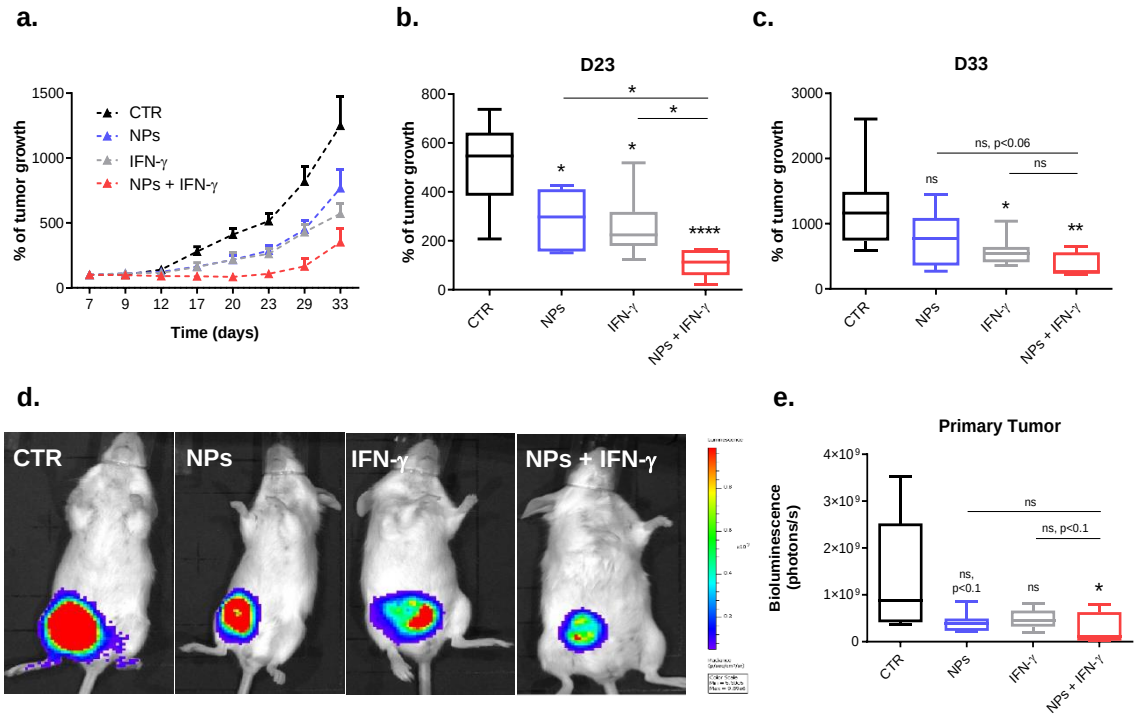

Figure 4. Ch/γ-PGA NPs plus IFN-γ decreases 4T1 primary tumor progression. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+IFN-γ) and left 10 days without treatments. Non-treated animals were used as control. **a.** Tumor volume (mm^3) was measured using a caliper and the growth kinetics were normalized to the initial tumor volume for each animal. **b. and c.** Relative (%) tumor growth was measured at the end of the treatments at day 23 (D23) and at the end of the experiment (D33). Data show the mean ± SEM. **d.** Mice were bioluminescence imaged to monitor tumor progression. Images were taken after 20 minutes after intraperitoneal injection of luciferin. Representative images from Day33 are evidenced. **e.** Quantification of total photon flux (the number of photons/second, p/s) at the tumor region. Median is represented by the horizontal line inside the box plots. Data is representative of at least 8 animals per group. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^{*}p<0.05$, $^{**}p<0.01$ and ****p<0.0001 relative to non-treated control or single treatments (NPs or IFN-γ)).

3.5 Ch/γ-PGA NPs plus IFN-γ treatment decreases the splenomegaly

The 4T1 orthotopic model was described to induce a leukemic reaction associated to granulocytosis and splenomegaly, following the injection of 4T1 cells on the mammary fat pad of BALB/c mice [40]. In agreement, we observed that the spleen weight from non-treated 4T1 tumor bearing mice had increased 7-fold by four weeks post tumor transplant, in comparison to non-tumor bearing animals (data not shown). Animals treated with NPs exhibited decreased spleen weight (0.49 ± 0.03 g, $p<0.051$) while the effects of IFN-γ treatment were negligible (0.66 ± 0.03 g), in comparison to control non-treated tumor bearing animals (0.71 ± 0.10 g). Importantly, NPs plus IFN-γ treatment significantly induced

a decrease on spleen weight (0.12 ± 0.01 g) in comparison with control and single-treated animals, probably due to the inhibition of granulocytic hyperplasia (Fig. 5a,b). The histology of the spleen is being currently analyzed to complement our study. Additionally, myeloid colony-stimulating factors, namely GM-CSF, M-CSF and G-CSF, previously described to be associated with leukemic reaction in the spleen of 4T1 tumor bearing mice [43], were quantified in the mouse serum. Curiously, no evident alterations were observed in the levels of GM-CSF and M-CSF in animals treated with NPs plus IFN-γ comparing to control animals (Fig. 5c,d). Regarding G-CSF, control animals had increased 19-fold expression in comparison to non-tumor bearing mice (data not shown). Interestingly, NPs- or IFN-γ-treated animals presented higher levels of G-CSF comparatively to control animals, while those treated with the combinatorial treatment decreased the levels in comparison with single treatments, but the levels were similar to the control (Fig. 5e). Altogether, these results suggest that NPs plus IFN-γ treatment impaired splenomegaly condition, but no major evidences were provided by the myeloid colony-stimulating factors analyzed.

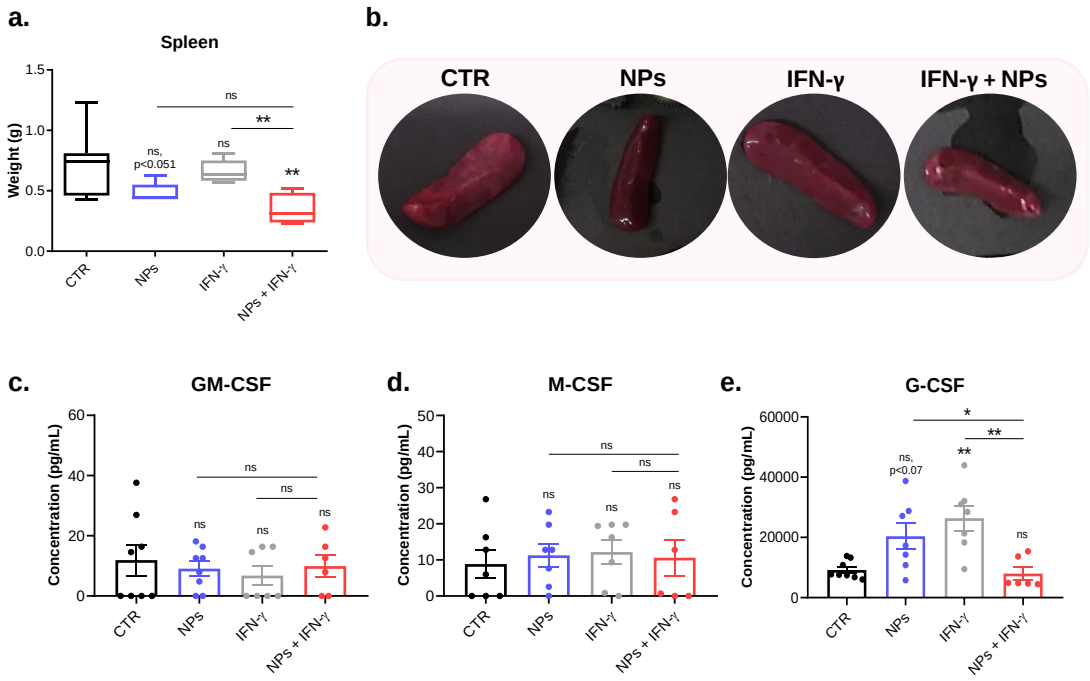

Figure 5. Ch/γ-PGA NPs plus IFN-γ impacts on spleen weight. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+ IFN-γ) and left 10 days without treatments. Non-treated animals were used as control **a.** Spleen weight (g) was measured at the experimental endpoint, day 33 (Day33). **b.** Representative images of mice spleen. **c-e.** Serum was analyzed at day 33 through multiplex immunobead assay technology, considering distinct macrophage chemoattractant factors. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 6 animals per group. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^{*}p<0.05$ and $^{**}p<0.01$ relative to non-treated control or single treatments (NPs or IFN-γ)).

3.6 Ch/γ-PGA NPs plus IFN-γ treatment increases CD4 T cells in the spleen

4T1 tumor progression is described to be followed by a decrease of T cells in the spleen and an increase in granulocytes over time [43]. To understand the impact of NPs plus IFN-γ treatment in the immune response, we dissociated spleens from different groups at day 33 into single suspensions and analyzed, by flow cytometry, both lymphoid and myeloid populations. Briefly, $CD4^+$ and $CD8^+$ T cells were gated on $CD45^+CD3^+$ T cells. T cells-expressing IFN-γ or PD-1 were gated on $CD4^+$ or $CD8^+$ T cells and FoxP3-expressing cells were gated on $CD4^+$ T cells (Fig. 6a). We observed that NPs or IFN-γ treatments had a negligible impact on T cells in the spleen (Fig. 6b-i) whereas the combination of NPs with IFN-γ increased the percentage of $CD4^+$ T cells in the spleen, in comparison to the control and to NPs-treated group (Fig. 6c). Additionally, these cells were more prone to express IFN-γ than cells from control or IFN-γ-treated groups (Fig. 6d), while no alterations were observed $CD3^+CD4^+$ T cells-expressing FoxP3 (Fig. 6e). The percentage of $CD8^+$ T cells in the spleen at day 33 was lower than that of $CD4^+$ T cells, and no alterations were observed in treated groups (Fig. 6f). Nevertheless, $CD8^+$ T cells-expressed higher IFN-γ levels, in comparison to control or to IFN-γ-treated animals (Fig. 6g). Interestingly, no differences were found in the expression of PD-1 by $CD4^+$ T and $CD8^+$ T cells in the different groups (Fig. 6h,i). Overall, these results suggest that the combination of NPs with IFN-γ increased the T helper 1 ($CD3^+CD4^+$IFN-γ^+ cells) response in the spleen, which can contribute to decrease tumor progression.

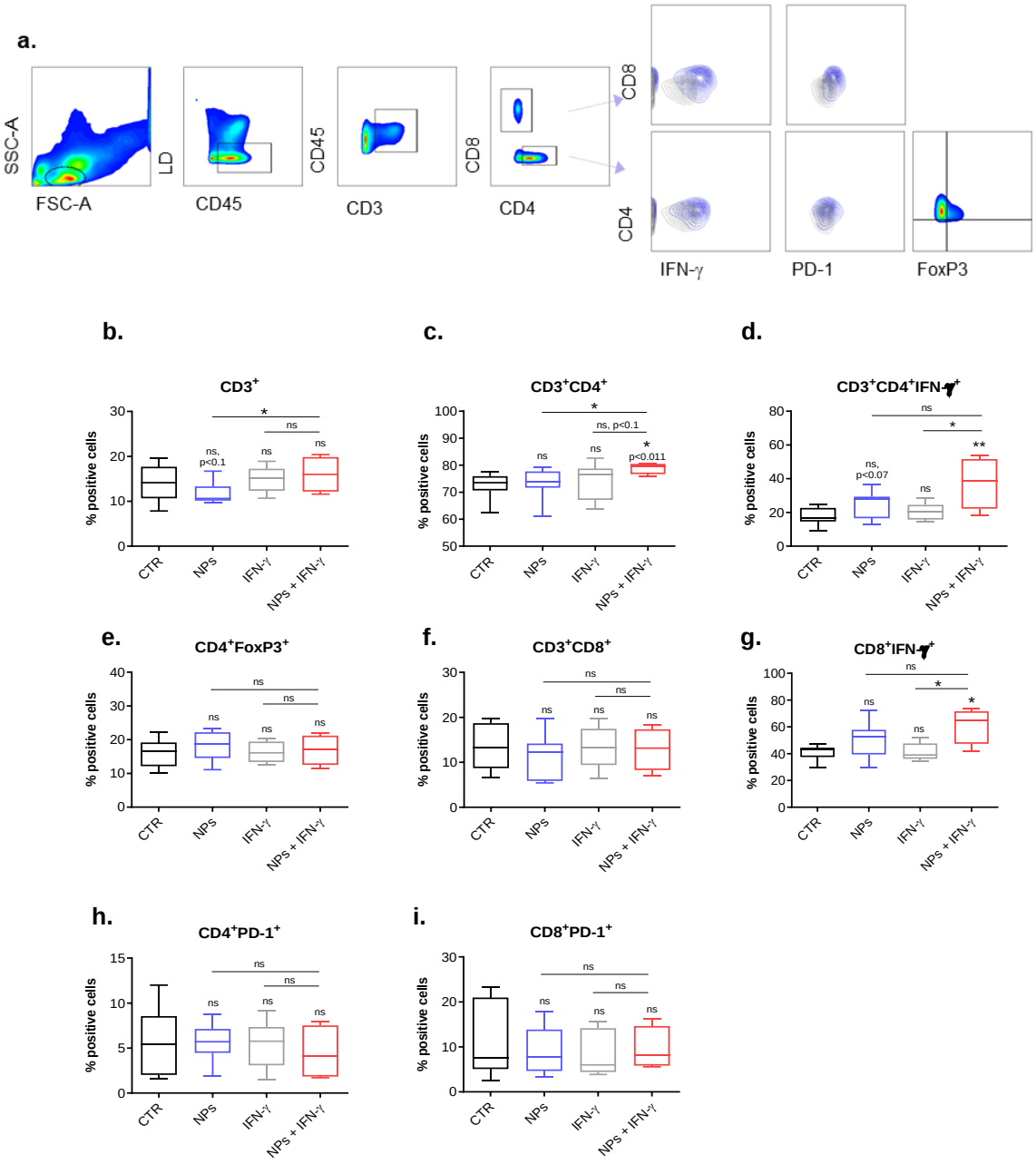

Figure 6. Ch/γ-PGA NPs plus IFN-γ impacts on spleen lymphoid cellular dynamics. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+ IFN-γ) and left 10 days without treatments. At day 33, spleens were collected and processed for flow cytometry analysis. **a.** The pseudocolor plots indicate the gate strategy of T cells. $CD45^+$Live Dead $(LD)^-$ cells were gated on single cells (FSC-A vs SSC-H). $CD3^+$ were gated on $CD45^+LD^-$ cells. $CD3^+CD4^+$, $CD3^+CD8^+$ T cells were gated on $CD3^+$ cells. IFN-γ and PD-1-expressing T cells were gated on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. FoxP3-expressing cells were gated on $CD3^+CD4^+$ T cells. **b-i.** The percentage of positive cells was determined. Data is representative of at least 7 animals per group. Median is represented by the horizontal line inside the box plots. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^*p<0.05$, $^{**}p<0.01$ relative to non-treated control or single treatments (NPs or IFN-γ)).

3.7. Ch/γ-PGA NPs plus IFN-γ impacts myeloid populations and B cells in the spleen

Previous studies showed that 4T1 tumor progression was followed by an increase in the percentage of $CD45^+$ cells in the spleen, mainly $Gr\text{-}1^+$ cells, which was correlated with increased spleen weight [43]. Briefly, $CD11b^+$ cells were gated on $CD45^+LD^-$ cells. Macrophages, defined with F4/80 and Ly6C markers, and granulocytic populations, defined with Ly6C and Ly6G markers, were gated on $CD11b^+$ cells. B cells were additionally identified as $CD45^+CD11b^-MHCII^+$ cells (Fig. 7a). Through the analysis of myeloid cells in the spleen, we observed a slight increase in $CD45^+$ cells in the animals treated with the combinatorial treatment of NPs plus IFN-γ, in comparison to control and to single treated groups (Fig. 7b). Interestingly, the percentage of myeloid cells ($CD45^+CD11b^+$ cells) was significantly decreased in the animals treated with the combination of NPs plus IFN-γ, in comparison with control and IFN-γ-treated group, almost reaching statistical significance for the NPs-treated group ($p<0.07$) (Fig. 7c). Specifically, macrophages population expressing F4/80 and Ly6C increased in relation to control and to IFN-γ-treated animals (Fig. 7d). Similarly, monocytic-like myeloid-derived suppressor cells (M-MDSC, $Ly6C^+Ly6G^-$ cells) were enhanced in the group treated with the combination of NPs plus IFN-γ, in comparison to control animals, but no differences were evidenced in relation to single-treated groups (Fig. 7e). Interestingly, granulocytic cells that are in high percentage in the spleen presented different dynamics. While granulocyte-like myeloid derived suppressor cells (G-MDSC, LyC^-Ly6G^+ cells) were significantly decreased in the spleen of animals treated with the combination of NPs plus IFN-γ (Fig. 7f), neutrophils ($Ly6C^+Ly6G^+$ cells) were increased (Fig. 7g). Regarding B cells, combination of NPs with IFN-γ slightly increased the percentage of B cells in comparison to control animals ($p<0.06$) while was significantly increased in relation to single-treated animals (Fig. 7h). Overall, these results indicate that NPs plus IFN-γ interfere with myeloid and B cell response in the spleen, by reducing the myeloid cells and slightly increasing the B cells, which could favor a better outcome in animals treated with the combinatorial treatment.

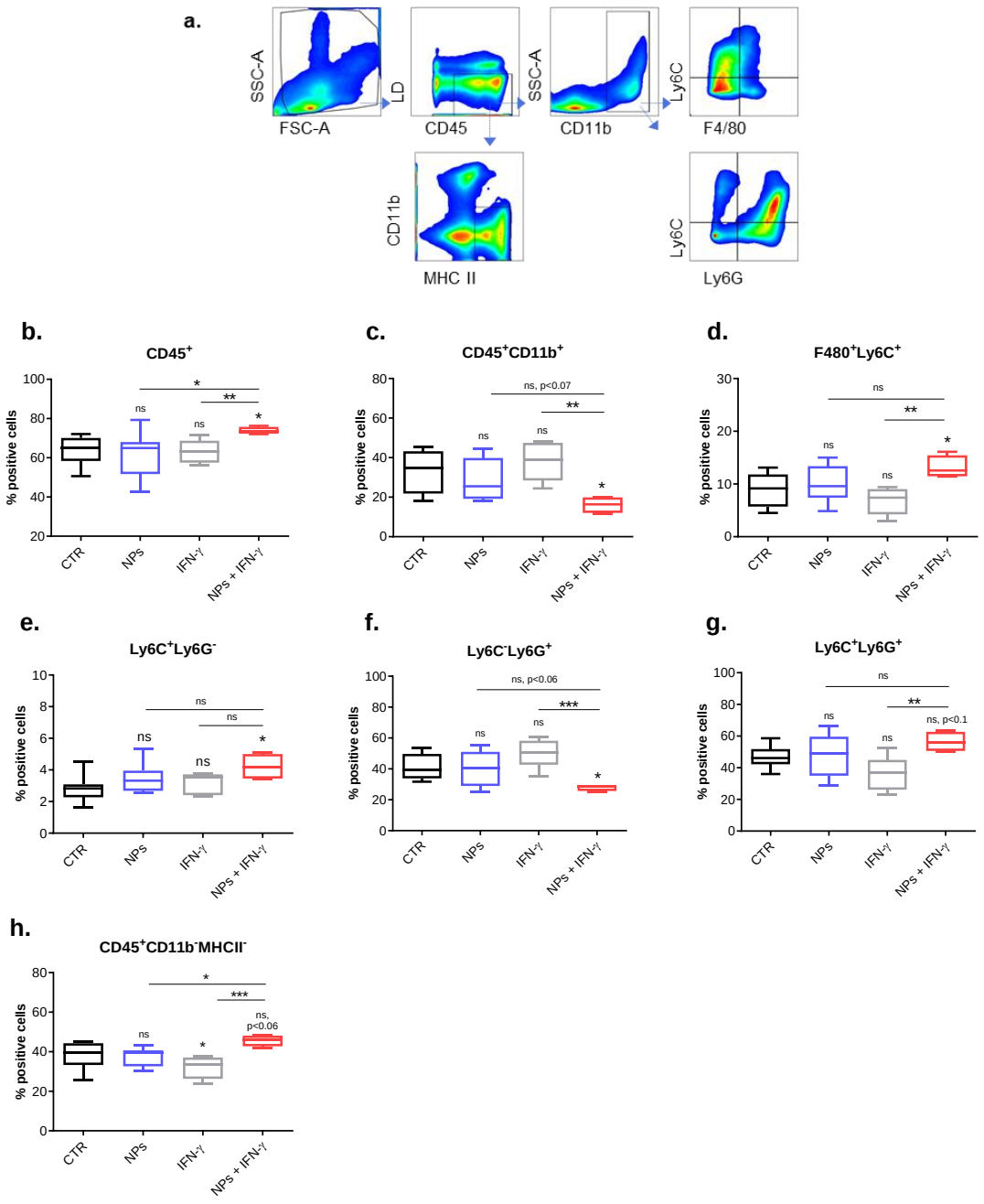

Figure 7. Ch/γ-PGA NPs plus IFN-γ impacts on spleen myeloid populations. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs, IFN-γ or with the combination of both (NPs+ IFN-γ) and left 10 days without treatments. At day 33, spleens were collected and processed for flow cytometry analysis. **a.** The pseudocolor plots indicate the gate strategy of myeloid and B cells. $CD45^+$Live Dead $(LD)^-$ cells were gated on single cells (FSC-A vs SSC-H). $CD11b^+$ cells were gated on $CD45^+LD^-$ cells. $F4/80^+Ly6C^+$, $Ly6C^+Ly6G^-$, $Ly6C^-Ly6G^+$, $Ly6C^+Ly6G^+$ cells were gated on $CD11b^+$ cells. $CD45^+CD11b^-MHCII^+$ were gated on single cells. **b-h.** The percentage of positive cells was determined. Data is representative of at least 7 animals per group. Median is represented by the horizontal line inside the box plots. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ relative to non-treated control or single treatments (NPs or IFN-γ)).

3.8 Ch/γ-PGA NPs plus IFN-γ treatment slightly impacts systemic immune response

To characterize the immune mediators involved in the systemic response to the different treatments, the serum was analyzed through a multiplex analysis for a commercial panel of mouse cytokines and chemokines. We observed that 4T1 tumor inoculation induced higher levels of cytokines with immunosuppressive or protumoral properties, namely IL-3, IL-4, IL-6 and IL-10 in comparison to non-tumor bearing animals (Fig. 8a), whereas the pro-inflammatory cytokines IL-12p40, IL-1β, TNF-α and IFN-γ were not significantly affected (Fig. 8b).

Regarding the impact of the treatments on the systemic response, we generally observed a slight decrease in the levels of immunosuppressive cytokines in the serum of animals treated with the combination of NPs plus IFN-γ, just reaching, however, statistical significance for IL-4 levels (Fig. 8c-f). Regarding cytokines with pro-inflammatory activities, we did not observe major alterations on IL-12/IL-23p40, TNF-α and IFN-γ in NPs plus IFN-γ-treated animals, but the levels of IL-1β were significantly decreased (Fig. 8 g-j). Altogether, these results support that the combination of NPs with IFN-γ attenuated the immunosuppressive environment without increasing the classical pro-inflammatory cytokines which may have contributed to a better control of tumor progression.

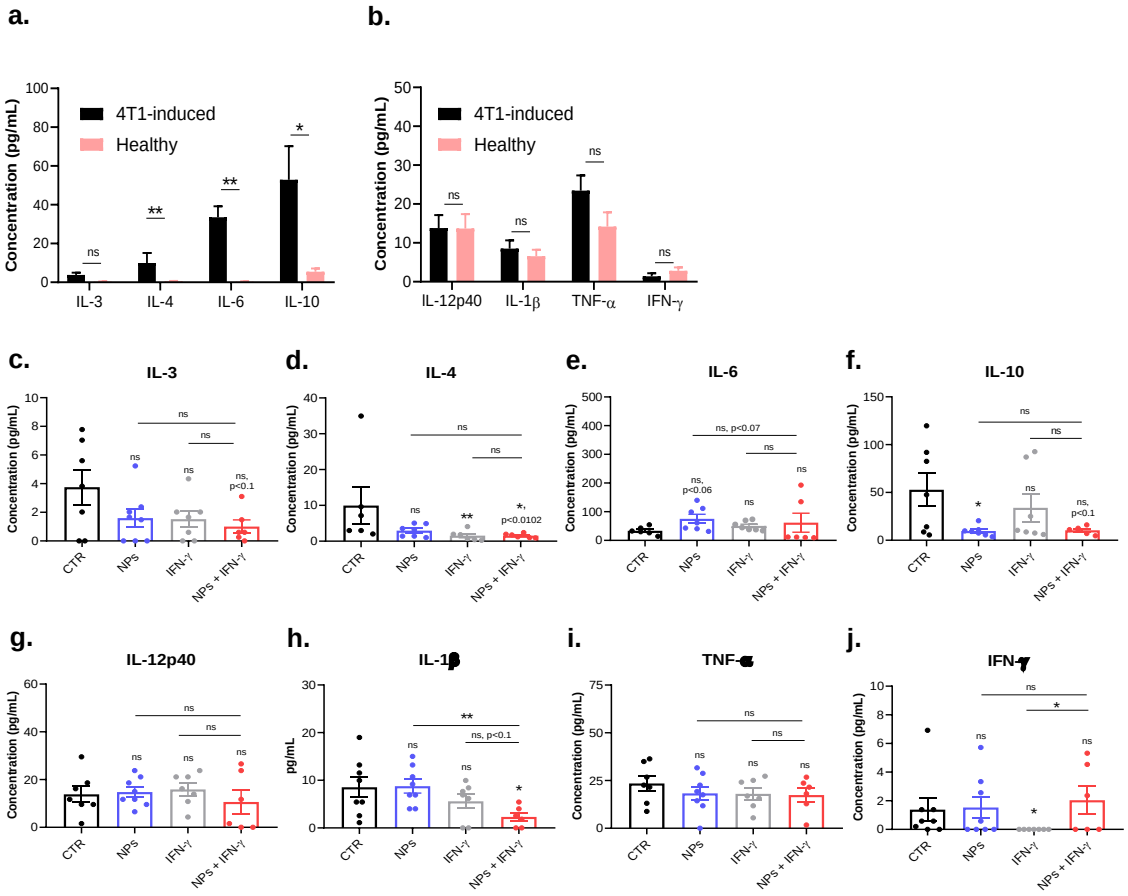

Figure 8. Ch/γ-PGA NPs plus IFN-γ attenuates the immunosuppressive environment. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+ IFN-γ) and left 10 days without treatments. Non-treated animals were used as control. Blood of mice was collected at the end of experiment (day 33) and processed for serum collection. **a. and j.** Serum was analyzed through multiplex immunobead assay technology. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 6 animals per group. **a. and b.** Serum from healthy animals was used as control. Comparisons were performed using the unpaired Mann-Whitney test (*p<0.05, **p<0.01). **c-j.** All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test (*p<0.05, **p<0.01 relative to non-treated control or single treatments (NPs or IFN-γ)).

3.9. Ch/γ-PGA NPs plus IFN-γ treatment decreases lung metastasis burden

The 4T1 orthotopic model is highly malignant with frequent lung and liver metastasis [40]. To evaluate the impact of combinatorial treatment on disease progression and metastasis formation, animals were bioluminescence imaged for almost five weeks, but we did not observe bioluminescence signal in the lungs or liver *in vivo* (data not shown). Therefore, at day 33, we collected the lungs and the liver to image *ex vivo.* Interestingly, animals treated with NPs or with IFN-γ presented a similar signal ($2.6x10^5 \pm 1.1x10^5$ p/s or $2.4x10^5 \pm 5.5x10^4$

p/s, respectively) in comparison with control animals ($3.1x10^5 \pm 1.3x10^5$ p/s), while animals treated with the combination of NPs plus IFN-γ experimented a significant decrease in the bioluminescent signal ($1.2x10^5 \pm 2.2x10^3$ p/s) (Fig. 9a,b). No statistical differences were observed in the liver (data not shown). We are currently analyzing the hematoxylin and eosin staining of liver and lungs, to complement these bioluminescence results. Additionally, we quantified the production of molecules associated with lung metastasis, namely CCL4 and VEGF, in the serum at day 33, and no differences were observed (Fig. 9c). Overall, the animals treated with the combination of NPs plus IFN-γ experimented a reduced primary tumor growth and, accordingly the bioluminescence imaged, a reduced lung metastases burden.

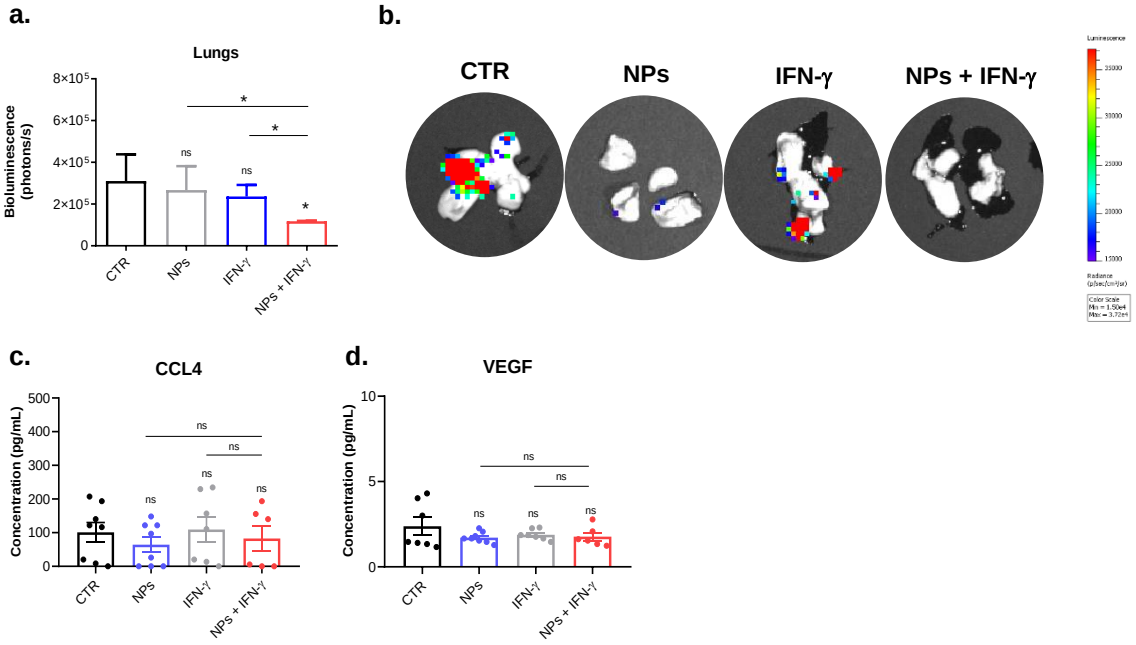

Figure 9. Ch/γ-PGA NPs plus IFN-γ decreases lung metastasis burden. BALB/c mice injected with 4T1 cells on the mammary fat pad were treated from day 7 until day 23 with 6 doses of Ch/γ-PGA NPs (NPs), IFN-γ (IFN-γ) or with the combination of both (NPs+ IFN-γ) and left 10 days without treatments. Non-treated animals were used as control. Animals were bioluminescence imaged using a IVIS Lumina II every week to control tumor progression and metastasis formation. At day 33 animals were euthanized and lungs were recovered for bioluminescence imaging *ex vivo*. **a.** Bioluminescence at the lungs was determined by drawing regions of interest (ROIs) and expressed in total flux (photon/s), through IVIS software. **c-d.** Serum was analyzed through multiplex immunobead assay technology. Outliers were calculated through ROUT method (Q=1%) and removed from the analysis. Data show the mean ± SEM and it is representative of at least 6 animals per group. All comparisons were performed using the Kruskal Wallis test followed by Dunn's multiple comparison test ($^*p<0.05$ relative to non-treated control or single treatments (NPs or IFN-γ)).

4. DISCUSSION

While several clinical trials failed to demonstrate the beneficial clinical effects of single IFN-γ therapy, the most recent clinical studies have explored the potential of IFN-γ as adjuvant treatment to conventional therapy or immunotherapy. The idea of combining different treatments to improve patient objective cancer response is not new. The combinatorial therapy is a cornerstone of cancer therapy, with the radiotherapy, chemotherapy and targeted therapy being combined for many years. The novelty is the combination with another therapeutic modality, the direct modulation of the immune system. This was achieved with the introduction of immunotherapies for cancer treatment, in 2011. Results from preclinical studies promise a great advantage of conventional therapies being combined with immunotherapy, for example the radioimmunotherapy, which is inherently limited in their application to the clinical setting until robust clinical trials determine the appropriate approach to combine different modalities in patients.

The potential of IFN-γ as adjuvant for cancer therapy is still unknown. Preclinical models highlight the importance of IFN-γ signaling for an effective antitumor response and for response to anticancer therapy [44-46], while a recent study (in peer-review) showed that the combination of IFN-γ with anti-epidermal growth factor receptor 2 (HER2) antibody further decreased primary tumor growth in a breast tumor model, followed by a decrease in CD8 T cells, Gr1 and PD-L1 expression [47]. Although further preclinical studies are required to test the adjuvanticity of IFN-γ to current anticancer treatments, this cytokine is being currently tested in clinical trials as single therapy (NCT01957709) or in combination with conventional therapy (NCT03112590, NCT00024271), immunotherapy (NCT02614456, NCT03063632) and autologous monocytes administration (NCT02948426). Looking back, some *in vitro* studies have demonstrated that the combination of TLR ligands with IFN-γ potentiate its tumoricidal activity and synergizes for the induction of a M1-like profile [22, 29, 48]. In fact, innate immune stimulators, including TLR agonists, are under active investigation in the cancer field. TLR agonists appears as appealing agents since they trigger a strong innate immune activation and can break the immunosuppression and tolerance at the tumor microenvironment [49, 50]. Currently, three TLR agonists are approved by Food Drug Association (FDA) for cancer prevention and treatment: the bacillus Calmette-Guérin (BCG), monophosphoryl lipid A (MPL) and imiquimod [51].

Here, we tested the potential of immunomodulatory and tumoricidal IFN-γ with the previously described immunostimulatory Ch/γ-PGA NPs to repolarize IL-10-stimulated

macrophages and to reprogram the tumor microenvironment in 4T1 breast tumor model. This therapeutic combination has never been explored before, and present data provide the proof of concept that NPs and IFN-γ can elicit together a significant antitumor immunity. By analyzing the impact on human monocytes-derived macrophages, we observed clearly a different impact of NPs and IFN-γ on macrophages repolarization. While IFN-γ upregulates CD86 expression, NPs downregulate CD163 expression, and when they were combined both effects are maintained and seemed potentiated. Additionally, we verified that NPs stimulation induced pro-inflammatory cytokines production while IFN-γ do not. Curiously, this effect was not potentiated by the combination of NPs plus IFN-γ, in opposite to what was previously reported, where the combination of IFN-γ with TLR ligands enhanced pro-inflammatory cytokines production by bone marrow-derived macrophages (BMDM) [29]. We tested the impact of this strategy in the tumoricidal activity of macrophages by incubating RKO cancer cells with conditioned medium collected after macrophage stimulation. IL-10-stimulated macrophages did not present tumoricidal ability as expected, but the stimulation with NPs plus IFN-γ seems to render tumoricidal macrophages and this effect was mediated by IFN-γ. These results are in contrast with what was recently reported using BMDM which demonstrated that macrophages stimulated with IFN-γ had no inhibitory effect on tumor cell growth. Still, the macrophages source and the IFN-γ concentration were different [29]. Altogether, these *in vitro* data suggest that IFN-γ and NPs synergize to revert a macrophage immunosuppressive phenotype (IL-10-induced) towards an immunostimulatory and tumoricidal profile (M1-like). These results will benefit from a transcriptome analysis to help us at understanding which genes and pathways are being involved in each activation.

Considering the *in vitro* results, the potential of the combinatorial treatment was assessed in 4T1 breast orthotopic tumor model, characterized by poor immunogenicity and by closely mimicking the human disease. We demonstrated, for the first time, that adjuvant NPs combined with IFN-γ improved primary tumor growth control. This was primarily reflected by increased T helper 1 and neutrophils-mediated response, followed by a massive decrease in immunosuppressive G-MDSC in the spleen. Systemically, no major alterations were observed unless the decrease of protumoral IL-4, and of IL-1β. Importantly, the levels of IL-10 and IL-3 which are associated with protumoral activities were also slightly reduced. Notably, animals treated with NPs plus IFN-γ presented lower bioluminescence signal in the lungs comparing to the control and single-treated animals, which suggests a lower metastatic burden. In support of these results it is well described that 4T1 tumors are constituted by a high percentage of myeloid cells (Gr1b+ CD11b+) which can inhibit T cell activities [52] and promote lungs metastases formation [53]. Additionally, the depletion of

G-MDSC using anti-Ly6G resulted in suppression of pulmonary metastasis [53]. We are currently analyzing the metastas in the lungs and liver and will explore the existence of a probable correlation between the metastatic burden and G-MDSC in these organs. Additionally, other researchers identified a “breast metastatic gene signature” composed by *S100A9*, *MMP8*, *S100A8*, *FPR1*, *CCL3* and *TGFB2* that were particularly upregulated by lung-derived G-MDSC from metastatic 4T1 primary tumors [53]. At this point, we only analyzed CCL3 and CCL4 in the mouse serum and no differences between control and treated animals were found. Curiously, the single treatments were not enough to promote T cell activation neither impact on the myeloid populations, probably due to the well-established immunosuppressive environment created by the established 4T1 tumor, highlighting the importance of combinatorial therapy. Importantly, the combination of NPs with IFN-γ blocked the tumor growth during treatment (from day 7 to day 23), while single treatments delayed the tumor growth, which indicates that both treatments synergized to block the tumor progression, probably by eliciting a stronger antitumor immune response and decreasing the immunosuppressive cell players. It would be interesting to characterize both primary tumors and spleen immune response at this time-point, to follow the dynamic of immune cells, and to evaluate if the changes observed at day 33 would be more pronounced at day 23. Still, when the treatments were stopped, the tumors from all treated groups, including from the combinatorial therapy, grew, suggesting the necessity of a sustained therapy.

Overall, we demonstrated in the present study that is possible to achieve a systemic therapeutic immune response against a poorly immunogenic mammary carcinoma through the combination of Ch/γ-PGA NPs with IFN-γ administered locally. This low-cost immunotherapy-like strategy has the advantage of immunomodulatory Ch/γ-PGA NPs can be combined with targeted antibodies, be used as carriers for tumor antigens, and be delivered locally without pathological reaction.

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CHAPTER VII

Chitosan/poly(γ-glutamic acid) nanoparticles incorporating IFN-γ potentiate the immune response in the context of colorectal cancer

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ABSTRACT

IFN-γ therapy has been approved by the Food and Drug Administration (FDA) for the treatment of chronic granulomatous disease and severe malignant osteopetrosis. Despite the promising IFN-γ-based therapeutic applications, its limited success in clinical trials is related with limitations inherent to its molecular properties and with the difficulties to deliver it locally or with the adequate periodicity to achieve a therapeutic effect.

We have previously shown that chitosan (Ch)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) are immunostimulatory, impairing colorectal cancer cell invasion. Ch is a biocompatible cationic polysaccharide extensively studied and already approved for biomedical applications while γ-PGA is a poly(amino acid), biodegradable and negatively charged. Here, we evaluated the potential of Ch/γ-PGA NPs as vehicles for IFN-γ and their ability to modulate immune cells' phenotype.

In this study, Ch/IFN-γ/γ-PGA nanoparticles (IFN-γ-NPs) prepared by co-acervation method, presenting a size of approximately 180 nm and a low polydispersion index, were tested for their immunomodulatory activity. These IFN-γ-NPs induced an immunostimulatory profile on dendritic cells (DCs) with increased cell surface costimulatory molecules and secretion of pro-inflammatory cytokines, including IL-6, IL-12p40 and TNF-α. IFN-γ-NPs also modulated IL-10-stimulated macrophage profile, increasing their ability to secrete the pro-inflammatory cytokines IL-6, IL-12p40 and TNF-α. Concomitantly, these phenotypic alterations enhanced T cell proliferation. In addition, the ability of DCs and macrophages to induce colorectal cancer cell invasion was hampered in the presence of IFN-γ-NPs. Although the major observations were Ch/γ-PGA NPs-mediated, the incorporation of IFN-γ on NPs potentiated the expression of CD40 and CD86, and the impairment of colorectal cancer cell invasion. This work bridges the previously reported immunostimulatory Ch/γ-PGA NPs capacity, with their potential as carriers for immunomodulatory molecules, like IFN-γ, opening new perspectives for their use in clinical settings.

1. INTRODUCTION

The tumor microenvironment is a heterogeneous and structurally complex tissue where immune cells have been assumed as detrimental players of tumor progression [1-3]. Several studies have evaluated the relevance of leukocyte infiltration in human tumors and identified several immune cell types that elicit immunosuppressive activities, including anti-inflammatory macrophages, immature or dysfunctional dendritic cells (DCs), myeloid-derived suppressor cells (MSDCs), regulatory T cells (Tregs) and T helper 2 (Th2) cells [4]. Although all these cell types have important roles in tumor progression, myeloid leucocytes have been recognized as the major tumor-promoters [5].

Specifically, macrophages are the patrolling sentinels of the immune system and are responsible for the recognition and elimination of danger signals to the host. Although macrophages have the potential to recognize and kill tumor cells – immunosurveillance - and to elicit antitumor immunity, different studies evidenced that tumor-associated macrophages (TAMs), when chronically activated, are dictators of tumor development by promoting tumor cell proliferation and survival, angiogenesis, invasion and metastasis, while dampening effective T cell responses [6-9]. Since macrophages can experience functional reprogramming in response to different stimuli, reflecting their extreme phenotypic and functional plasticity, they are attractive therapeutic targets [10, 11]. Accordingly, the most recent macrophage phenotype classification comprises a wide spectrum where M1- and M2-polarized macrophages are usually accepted as extremes of a universe of functional states [12, 13]. M1-like macrophages are generally induced by interferon-gamma (IFN-γ), tumor-necrosis factor alpha (TNF-α) and by TLR agonists, as lipopolysaccharides (LPS). They are associated with pro-inflammatory, antimicrobial and antitumoral activities and with high secretion of pro-inflammatory cytokines, as IL-6, IL-12, IL-23 and TNF-α; nitric oxide, reactive oxygen intermediates and low IL-10 levels [14]. Inversely, M2-like macrophages may be induced by IL-4, IL-10, TGF-β, IL-13 and by glucocorticoids hormones. They generally secret low levels of IL-6 and IL-12, high levels of IL-10 and are associated with tissue repair, proteolysis, angiogenesis and tumor progression [15-18]. Although several macrophage phenotypes are described within the tumor microenvironment, TAMs are frequently compared to M2-like macrophages [19, 20]. Recently, our group described that IL-10-stimulated macrophages promote gastric and colorectal (CRC) cancer cell invasion, migration and proteolysis, through the phosphorylation of cancer cell epidermal growth factor receptor (EGFR) and EGFR-downstream signaling partners, and production of matrix metalloproteinases (MMPs) [21].

Myeloid DCs have been shown to have an important role in cancer immunosurveillance, tumor antigen presentation and consequently, in the priming and maintenance of the antitumor immune response [22]. These cells also present a high functional plasticity, depending on the microenvironment signals that can affect their differentiation, maturation and activation [23]. Frequently, DCs maturation, activation and their antigen presentation capacity are compromised at the tumor microenvironment, impairing antigen presentation capacity and favoring tumor immune escape [22].

Considering the crucial role of macrophages and DCs in eliciting an efficient antitumor immune response and their functional and phenotypic plasticity, several strategies targeting or reprograming these cells have been reported [24-29]. One of them is IFN-γ-based therapies, a cytokine with strong immunostimulatory and antitumor properties [30], which has been described to activate M1 macrophage differentiation, and to reverse the immunosuppressive and protumor role of human ovarian tumor-associated macrophages [31]. IFN-γ is secreted by a variety of immune cells, including activated-T cells, B cells, natural-killer (NK) cells and professional antigen-presenting cells (APCs), upregulates macrophage expression of major histocompatibility complexes (MHC), production of IL-12 and TNF-α, and orchestrates Th1-immune responses [32]. Currently, IFN-γ is approved by the Food and Drug Administration (FDA) for chronic granulomatous disease and severe malignant osteopetrosis [33]. In the oncological field, IFN-γ-based therapies have undergone several clinical trials, frequently in combination with other therapies such as immunotherapy and chemotherapy, for ovarian and peritoneal cancers, breast cancer, melanoma, and soft tissue sarcoma [30]. Nevertheless, the success of IFN-γ clinical use has been limited due to the need of administering high doses by systemic delivery, leading to significant toxicity and severe adverse effects [30, 34]. Additionally, IFN-γ has a short half-life requiring recurrent administrations [35]. These limitations encouraged the search for alternative delivery methods to maximize the therapeutic efficacy of this cytokine and minimize its toxicity. Such systems, aiming at a continuous localized delivery, include liposomes, polymer gels, and biodegradable microspheres [36-40]. Recently, our group has shown that IFN-γ can be successfully incorporated into polyelectrolyte multi-layered films (PEMs) of Chitosan (Ch)/poly(γ-glutamic acid) (γ-PGA) under mild conditions and efficiently revert the invasion of cancer cells, induced *in vitro* by IL-10-treated macrophages [41]. Ch and γ-PGA are non-toxic and biodegradable polymers appealing for biomedical applications. Ch is cationic polysaccharide at pH below 6.5 while γ-PGA is a hydrophilic poly (amino acid), negatively charged at pH above 2.2 [42, 43]. Ch and γ-PGA can assemble into the form of nanoparticles (NPs) by co-acervation method, as previously described [44].

Recently, we reported that Ch/γ-PGA NPs have the ability to reprogram IL-10-stimulated macrophages and immature DCs towards an immunostimulatory profile, enhancing T cell activation and proliferation, while decreasing colorectal cancer cell invasion [44]. Ch/γ-PGA NPs have also demonstrating to be successful and versatile delivery systems for hormones, chemokines, anti-inflammatory drugs and antibiotics [45-49], but the potential to deliver immunomodulatory cytokines is yet unknown. Here, we evaluated the potential of Ch/γ-PGA NPs to be used as an IFN-γ delivery system and characterized its synergetic effectiveness in modulating macrophages and DCs towards a pro-inflammatory and antitumor phenotype. The impact of stimulated macrophages and DCs on T cell proliferation and on cancer cell invasion was also investigated. This report provides a proof-of-concept for a future therapeutic strategy based on Ch/γ-PGA NPs as carriers for the immunomodulatory IFN-γ and their ability to target myeloid cells at the tumor microenvironment.

2. MATERIAL AND METHODS

2.1. Ch/γ-PGA NPs preparation

Ch/γ-PGA NPs were prepared through a co-acervation method, as we have previously described [44]. Ch (France-Chitine) was purified and characterized as described by Antunes *et al*. [50]. Ch with a degree of acetylation (DA) of 10.4±1.6%, as determined by Fourier transform infrared spectrometry (FTIR-KBr), and with a molecular weight (MW) of 324±27 kDa, as measured by size-exclusion chromatography, was used. γ-PGA (10-50 kDa; 99.5% purity) was produced from *Bacillus subtilis*, as described by Pereira *et al*. [51]. Ch/γ-PGA NPs were prepared at a molar ratio of 1:1.5 (mol Ch:mol γ-PGA). Solutions of Ch (0.2 mg/mL in 0.2 M AcOH) and of γ-PGA (0.2 mg/mL in 0.05 M Tris-HCl with 0.15 M NaCl) were combined by co-acervation method. Briefly, a γ-PGA solution was dropped into a Ch solution, through a 1 mL syringe associated to a syringe pump (KD Scientific Inc., Holliston, MA), at a constant speed (3.6 µL/s), under high stirring, at room temperature (RT). All solutions were adjusted to a pH of 5.0. For the synthesis of Ch/IFN-γ/γ-PGA NPs (IFN-γ-NPs), IFN-γ was added to the Ch solution, followed by the addition of γ-PGA by dropping. Recombinant Human IFN-γ (ImmunoTools) at 10 µg/ml was incorporated in Ch/γ-PGA NPs at molar ratios of 1:0.1:1.5, 1:0.25:1.5, 1:0.35:1.5, 1:0.5:1.5 and 1:0.7:1.5 (mol Ch:mol IFN-γ:mol γ-PGA), that corresponded to 43, 107, 150, 214, 300 ng/ml of IFN-γ (Fig. S1). NPs were then concentrated 10 times by centrifugation (13000 rpm, 30 min, 4ºC) followed by resuspension in pH 5.0 buffer solution, at one tenth of the initial volume. Concentrated NPs

were always used at the final concentration of 1% (v/v) to minimize pH alterations. The endotoxin levels in the NPs suspension were measured through the limulous amoebocyte lysate test (Lonza). These were 0.041 EU/mL which is considered negative according to the manufacturer's instructions (only positive if ≥ 0.125 EU/mL).

2.2. IFN-γ-NPs characterization

IFN-γ-NPs were characterized in terms of size, polydispersion index and zeta potential by dynamic light scattering (DLS), using a ZetaSizer Nano Zs (Malvern Instruments), equipped with a He-Ne laser (λ= 633 nm), as described elsewhere [46]. Every NPs batch produced was tested and 3 measurements per sample were acquired. The stability of NPs in 0.05 M Tris-HCl with 0.15 M NaCl buffer at 4 ºC was confirmed for 3 weeks through DLS analysis.

2.3. IFN-γ incorporation on NPs

For flow cytometry analysis, IFN-γ-NPs were first coupled to 4 µm size aldehyde/sulphate latex beads 4%(w/v) (Invitrogen). Sonicated beads were incubated with NPs at 1:2 ratio (v/v), overnight at RT and under mild agitation. For negative controls, Ch/γ-PGA NPs were used. For positive control, IFN-γ-coupled beads were used. Functional groups remaining on the beads were blocked by incubation with glycine 100 mM, for 30 min at RT, under mild agitation. Samples were immunostained with an anti-human IFN-γ PE-conjugated antibody (ImmunoTools), and samples were analyzed in a FACS Canto Cytometer (BD Biosciences). Results were analyzed using FlowJo software version 10 (TreeStar, Inc.).

2.4 IFN-γ release profile upon pH modification

IFN-γ-NPs were incubated in PBS at pH 6 and pH 7.4. At distinct time-points, the solution was recovered and centrifuged (60 min at 20 000 rpm) and IFN-γ concentration on the supernatants was determined by ELISA, according to manufacturer's instructions (Biolegend). The percentage of IFN-γ release at each time-point was calculated as: % IFN-γ released = [IFN-γ] supernatant/[IFN-γ] initial *100.

2.5 Ethics Statement

Human samples were obtained in agreement with the principles of the Declaration of Helsinki. Monocytes were isolated from surplus buffy coats from healthy blood donors, kindly provided by the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ), Porto, Portugal. Procedures were approved by the Hospital ethical committee

(Protocol reference 260/11). Blood donors provided informed written consent that the byproducts of their blood collections could be used for research purposes.

2.6. Human monocyte isolation and macrophage/DC differentiation

Human monocytes were isolated from buffy coats from healthy blood donors, as previously described by our lab [21]. Briefly, peripheral blood mononuclear cells (PBMCs) were collected from previously centrifuged buffy coats (30 min, 1200xg, RT, without brake), and incubated with RosetteSep human monocyte enrichment kit (StemCell Technologies), as described on manufacturer's instructions. This mixture was then diluted 1:1 in PBS supplemented with 2% heat inactivated fetal bovine serum (FBS, Biowest), layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged as previously. The enriched monocyte layer was collected and washed three times with PBS. Following this procedure, isolated cells were characterized by flow cytometry and over 80% were found to be CD14-positive. For monocyte-DC differentiation, $2.0x10^6$ monocytes were seeded on 9.6 cm^2 (6-wells plate) and differentiated for 5 days in complete RPMI1640 medium (supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin), supplemented with 50 ng/mL IL-4 and GM-CSF (ImmunoTools). IFN-γ-NPs (0.7 mg/mL) were added to DCs, (corresponding to 1% v/v) for additional 48 h, and controls were left unstimulated. LPS stimulation (25 ng/mL) was used as positive control. For monocyte-macrophage differentiation, $0.1x10^6$ monocytes were seeded on 3.8 cm^2 (24-wells plate), and cultured for 10 days in complete RPMI1640 medium, in absence of M-CSF. For IL-10 polarization, macrophages were stimulated with IL-10 (10 ng/mL, ImmunoTools) for additional 72 h. IFN-γ-NPs (0.7 mg/mL) were added to IL-10-stimulated macrophages at 1% (v/v), at 4 h post IL-10 stimulation, for 72 h.

2.7. Cell metabolic activity and viability

DC and macrophage cell cultures were observed daily under a light microscope (Olympus) from day 5 to day 7. Cell viability was measured using Annexin V Apoptosis Detection kit (BD Biosciences), allowing the identification of cell death by necrosis and apoptosis, at days 7 and 13, respectively. Briefly, Annexin V-FITC and propidium iodide (PI) staining were performed for 15 min at 4ºC. For each sample, $1.0x10^4$ cells were acquired using a FACS Canto Cytometer (BD Biosciences) and BD FACSDiva software. Results were analyzed using FlowJo software version 10 (TreeStar, Inc.). Macrophages and DC metabolic activity was determined by resazurin reduction assay. Briefly, 48 h or 72 h after IFN-γ-NPs addition, cells were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich) for 4 h at 37

°C and 5% CO_2 atmosphere. Fluorescence intensity on cell culture supernatants was then measured in a Synergy MX plate reader (590 nm, BioTek), and values were normalized relatively to unstimulated cells.

2.8. Western blot analysis

To understand whether IFN-γ-NPs activate IFNγ-receptor (IFNγR), phosphorylated STAT1 (pSTAT1) and total STAT1 expression levels were analyzed. For that, IL-10-treated macrophages were treated with IFN-γ-NPs, NPs and IFN-γ for 1 h. Then, cells were washed with PBS and lysed for 15 min on ice in RIPA buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA) supplemented with a mix of phosphatase and protease inhibitors (10 µg/mL NaF, 20 µg/mL Na_3VO_4, 10 µg/mL PMSF, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin and 50 µg/mL $Na_4P_2O_7$). After that, wells were scrapped, cell lysates collected and centrifuged (10 min, 14000 rpm, 4ºC). Supernatants were recovered and stored at -80ºC until protein quantification using the DC protein assay Kit (Bio-Rad Laboratories). Then, 20 µg of protein were dissolved in Laemmli buffer 4x (0.5 M Tris-HCl pH 6.8, 9.2 g SDS, 40 mL Glycerol, 5% β-mercaptoethanol, 5% bromophenol blue). Samples were subjected to denaturing conditions (5 min; 95ºC) and then separated by SDS-PAGE in 10% polyacrylamide gel. Resolved proteins were transferred into a nitrocellulose membrane (GE Healthcare) for 2 h at 100V. Next, membranes were blocked for 1 h with 5% non-fat milk in PBS- 0.1% Tween (PBST) to block nonspecific binding sites. After, membranes were briefly washed in PBST and then incubated with anti-human phospho-STAT1 (clone 58D6, 1:1000, Cell Signaling Technology), anti-human STAT1 (clone D1K9Y, 1:1000, Cell Signaling Technology) and anti-human tubulin (clone B-5-1-2, 1:10000, Sigma) antibodies overnight at 4 ºC. Membranes were washed 4 times in PBST for 5 min. Secondary antibodies used were HPR-conjugated anti-mouse (1:2500, Santa Cruz Biotechnology) and anti-rabbit (1:2500, Santa Cruz Biotechnology). Membranes were washed 6 times in PBST for 5 min. Finally, membranes were incubated Clarity Western ECL Substrate (BioRAD), and protein bands chemiluminescence determined with autoradiographic films. Films were scanned in a calibrated densitometer and band pixel density determined by image analysis, using ImageJ software.

2.9. DC and macrophage profile evaluation

For the analysis of cell surface receptor expression, DC and macrophage cultures were incubated with IFN-γ-NPs for 48 or 72 h, respectively. Afterwards, macrophages were

incubated with accutase (eBioscience) at 37 °C during 30 min and harvested by gently scrapping, whereas DCs in suspension were harvested. Cells were washed and resuspended in FACS buffer (PBS, 2% FBS, 0.01% sodium azide) and stained with specific conjugated fluorophore-antibodies, in the dark, for 45min at 4 °C. Macrophages were incubated with the following antibodies: anti-human CD14-APC (clone MEM-18), CD86-FITC (clone BU63), HLA-DR-FITC (clone MEM-12) (all from ImmunoTools) and CD163-PE (clone GHI/61; R&D Systems). In addition, DCs were immunostained with the following antibodies: anti-human CD1a-APC (clone HI149), CD11c-APC (clone BU15), HLA-DR-PE (clone MEM-12), CD86-FITC (clone BU63), CD40-PE (clone HI40a) (all from ImmunoTools) and CD83-FITC (clone HB15e; AbDSerotec). Isotype-matched antibodies were used as negative controls. After additional washes, $1x10^4$ cells were acquired on a FACS Canto Flow Cytometer and analyzed as previously. Median fluorescence intensity was calculated by subtracting the respective isotype control intensity.

2.10. DC and macrophage cytokine secretion profile by ELISA

Supernatants from DCs and macrophages collected after 48 h or 72 h of NPs stimulation, respectively, were assayed. IL-6, IL-12p40, IL-10 and TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA) TMB Development kit (Peprotech), according to manufacturer's instructions. IFN-γ levels were determined using BioLegend's LEGEND MAX™ Human IFN-γ ELISA Kit.

2.11. Analysis of T cell Proliferation

Monocytes plated at $1x10^5$ and $2x10^5$ cells per well for macrophage and DC differentiation, respectively, were stimulated with the different treatments for 24 h. Enriched lymphocyte populations were obtained from buffy coats of different donors by centrifugation over Lymphoprep (800 g, 30 min without brake), as previously described [44]. Following this procedure, flow cytometry analysis revealed that approximately 60% of isolated cells were $CD3^+$. To remove monocyte contaminants, PBMCs were washed with PBS and plated for 2 h at 37°C, to allow monocytes adhesion. The non-adherent lymphocyte fraction was collected and $1x10^7$ lymphocytes/mL were labelled with 1 mM CFSE (Invitrogen), in PBS (37°C, 15 min), followed by two washes (5 min, 2500 rpm) in PBS with 20% FBS. An enriched T cell suspension was resuspended in complete RPMI1640 medium and were added to macrophages at 8:1 ratio and to DCs at 10:1 ratio, respectively [44, 52, 53], or cultured alone with DC´s (IL-4 and GM-CSF) or macrophage (IL-10) differentiating cytokines and IFN-γ-NPs. Phytohemagglutinin (PHA, Sigma-Aldrich) stimulation was used as positive

control. After 7 days, cells were harvested and labelled with specific anti-human antibodies for CD3-APC (clone UCHT-1), CD4-PEDy647 (clone EDU-2) and CD8-PE (clone UCHT-4) (all from ImmunoTools), and further analyzed by flow cytometry. T cell division was determined by the extent of CFSE halving, on $CD3^+CD4^+$ cells and on $CD3^+CD8^+$ cells.

2.12. Matrigel Invasion assay

Non-activated and activated DCs, or non-polarized and IL-10-polarized macrophages (1×10^5), in contact with IFN-γ-NPs were added on the lower compartment while human colorectal cancer RKO cells (5×10^4) were seeded on the upper compartment of Matrigel-coated inserts with 8-µm pore size (BD Biosciences), as previously described [44]. After 24 h, filters were fixed for 15 min at RT with 4% paraformaldehdyde (Sigma-Aldrich). While non-invasive cells were scraped, invasive cells were counterstained with DAPI and visualized using a fluorescence light microscope (Leica). The total number of invasive RKO cells was counted, considering only the nuclei that completely passed through the pores.

2.13. Statistical Analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v5 (GraphPad-v5). Due to the limited number of donors included in test groups ($n<7$), a non-Gaussian distribution was considered. Therefore, the non-parametric Friedman test was used for paired comparisons between groups. Kruskal-Wallis test was used for non-paired comparisons between groups. Statistical significance was achieved when $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. RESULTS

3.1. Characterization of IFN-γ nanoparticles and their stability

First, IFN-γ-NPs were produced and extensively characterized. Based on IFN-γ isoelectric point, it was incorporated in the chitosan solution, both positively charged at pH 5. Then, γ-PGA negatively charged at pH 5, was dropped at constant rate into the previously prepared Ch/IFN-γ solution, promoting the establishment of electrostatic interactions (Fig. 1a). This strategy was based on previous work from our group that optimized the incorporation of IFN-γ into Ch/γ-PGA PEMs, by dissolving IFN-γ within cationic Ch solution prior to spontaneous assembly with anionic γ-PGA [41]. Furthermore, Ch and γ-PGA ratio was previously optimized to form stable NPs (molar ratio of 1:1.5 of Ch/γ-PGA) [51]. In order to encapsulate the maximum amount of IFN-γ, while maintaining the NPs nanosize and low polydispersion index (PdI), different amounts of IFN-γ (0.1, 0.25; 0.35, 0.5 and 0.7 mol) were combined with Ch and γ-PGA at constant molarities, 1 and 1.5 mol, respectively (Fig. S1a,b). The NPs with IFN-γ molar ratios 0.1, 0.25 and 0.35 of presented a small size, low PdI and positive zeta potential, without significant differences between concentrations. Therefore, we decided to select the Ch/IFN-γ/γ-PGA molar ratio of 1:0.25:1.5, since in previous studies a similar IFN-γ concentration was used [41]. In these conditions, IFN-γ-NPs had a size of 177 ± 6.4 nm, PdI of 0.19 ± 0.03 and zeta of 18.3 ± 1.1 mV. These parameters were similar to those observed for Ch/γ-PGA NPs without IFN-γ (183.0 ± 2.8 nm, 0.22 ± 0.01 PdI, 18.7 ± 0.8 mV) (Fig.1b,c). The stability of IFN-γ-NPs was confirmed, without significant alterations verified in their size and PdI after 3 weeks (Fig. 1d).

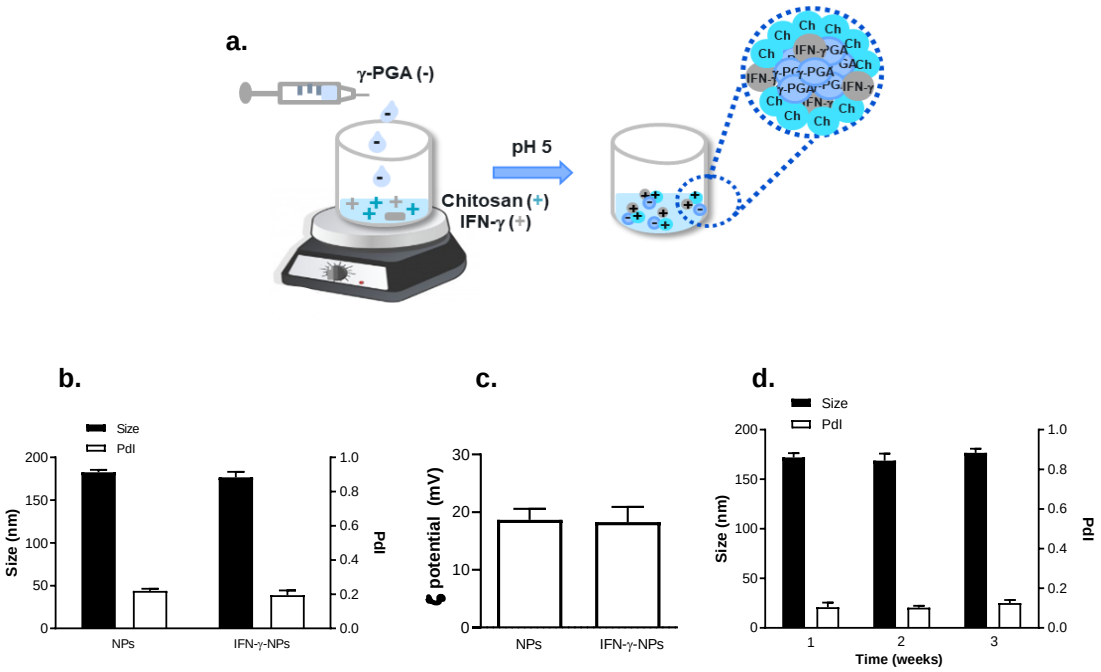

Figure 1 - **Characterization of Ch/IFN-γ/γ-PGA nanoparticles (IFN-γ-NPs). a.** Schematic representation of IFN-γ-NPs production. NPs were obtained by complex co-acervation method, driven by electrostatic interactions between chitosan and poly(γ-glutamic acid) (γ-PGA) at pH 5. γ-PGA solution was dropped to Ch solution under high stirring at room temperature, as previously described. NPs were characterized immediately after preparation in 0.05 M TrisHCl with 0.15 M NaCl buffer at pH 5.0 and 25°C. Different molar ratios of IFN-γ were tested for the synthesis of IFN-γ-NPs: IFN-γ at 1/0.25/1.5 molar ratio corresponds to a concentration of 107ng/mL. Ch/γ-PGA NPs were used as control. **b.** IFN-γ-NPs and control NPs size (nm) (black bars), polydispersity index (PdI) (white bars), **c.** ζ potential measurements were determined by dynamic light scattering (DLS). **d.** Size and PdI of IFN-γ-NPs, at 1/0.25/1.5 molar ratio, measured for 3 weeks to evaluate the IFN-γ-NPs suspension stability. Results are presented as mean ± SEM (n = 3). No statistical differences were found.

3.2. Evaluation of IFN-γ incorporation in Ch/γ-PGA nanoparticles and its release

To demonstrate that IFN-γ was incorporated into Ch/γ-PGA NPs, IFN-γ-NPs were coupled to latex beads, a technique frequently applied for exosomes characterization, and analyzed by flow cytometry [54]. IFN-γ-NPs-coupled beads presented a higher percentage of IFN-γ positive staining (51.9 ± 6.3%) (Fig. 2a) compared both to unstained beads and NPs-coupled beads, indicating IFN-γ incorporation in NPs. The percentage of IFN-γ positive staining for NPs-coupled beads (12.4 ± 2.1%) was higher than for unstained negative control, a background likely associated to Ch and γ-PGA autofluorescence.

IFN-γ release was then determined by ELISA after incubation of IFN-γ-NPs in PBS at pH 6 and pH 7.4. The results evidenced a burst release of IFN-γ at both pH's, reaching 97.4 ± 53.6 % and 41.8 ± 29.5 % at 2 h, for pH 6 and pH 7.4, respectively. IFN-γ-NPs released

about 70% lower amount of IFN-γ at physiological than acidic pH (Fig. 2b), which suggests that IFN-γ stability was affected at higher pH, possibly being degraded.

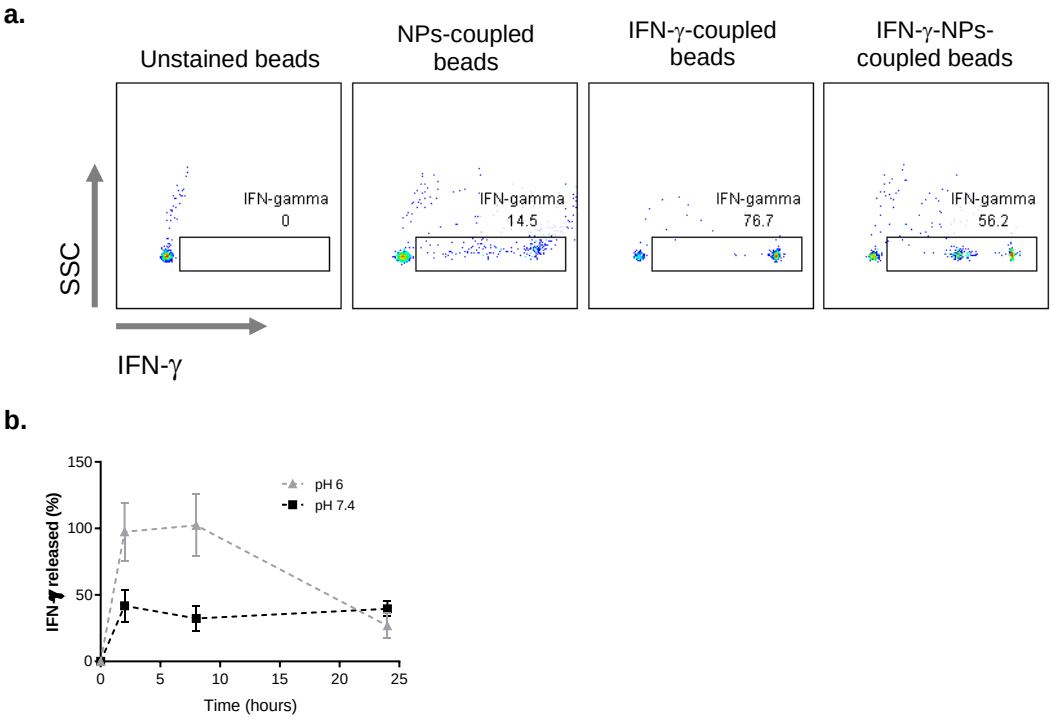

Figure 2 - IFN-γ incorporation and release on Ch/γ-PGA NPs. a. IFN-γ incorporation on Ch/γ-PGA nanoparticles was determined by flow cytometry. IFN-γ-NPs were first coupled to latex beads overnight at room temperature. Samples were immunostained with PE-conjugated anti-human IFN-γ and acquired using a FACS Canto Cytometer (n=2). **b.** IFN-γ release kinetics was analyzed by incubating IFN-γ-NPs in PBS, at pH 6 and 7.4, at 37°C. At different time points, the concentration of IFN-γ released was determined by ELISA. Results are presented as mean ± SEM (n = 3).

3.3. IFN-γ-NPs do not affect APCs viability nor metabolic activity

IFN-γ is well described as a strong modulator of immune cell phenotype and function [55]. However, its use has been limited due to its molecular properties and difficulties to deliver it locally, or with the adequate periodicity to achieve a therapeutic effect [33]. To evaluate the safety of IFN-γ-NPs on human DCs, IL-10-stimulated macrophages and their respective controls, cells were monitored for the existence of apoptosis-related morphological features, and their viability and mitochondrial metabolic activity investigated. Morphologically, no apoptotic signs, such as cell shrinkage, pyknosis or loss of membrane integrity, were observed in either cell populations, excluding possible high toxic effects (data not shown). Additionally, IFN-γ-NPs did not significantly increase the percentage of apoptotic or necrotic DCs or macrophages (Fig. 3a,b,c). The same was observed when DCs or IL-10-stimulated macrophages were treated with IFN-γ or NPs alone. Regarding the metabolic activity, as demonstrated by the resazurin assay, no significant differences were found between the distinct treatments (Fig. 3d). In summary, the IFN-γ-NPs treatment at pH 5 did not induce significant alterations on DC and macrophage morphology, viability and metabolic activity, suggesting that cell treatment with 0.7 mg/mL IFN-γ-NPs was suitable for following experiments.

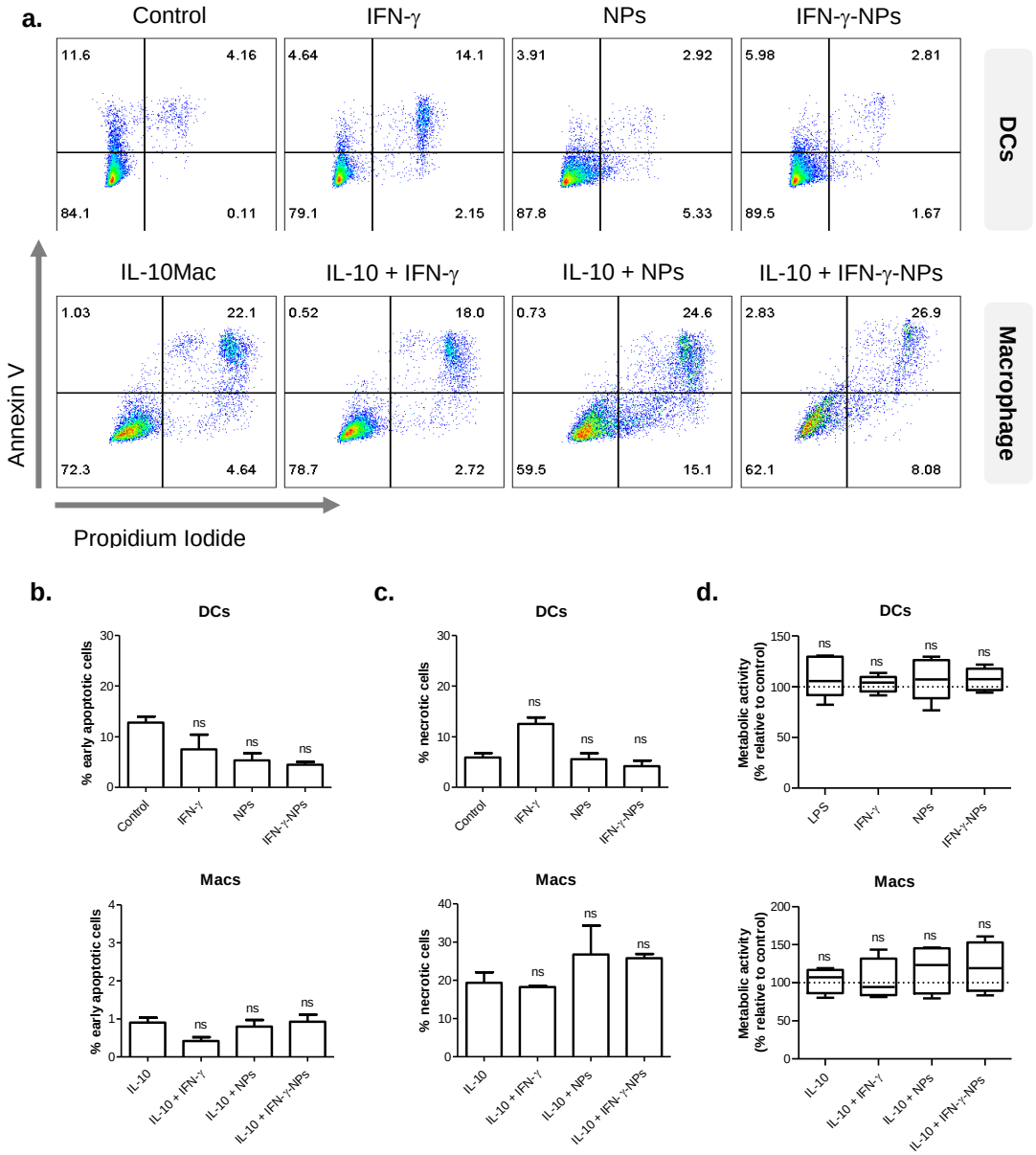

Figure 3. IFN-γ-NPs do not affect human DC and macrophage viability nor metabolic activity. Human differentiated DCs were cultured with IFN-γ, NPs or IFN-γ-NPs for 48 h. Control refers to unstimulated DCs. LPS was used as DCs' maturation positive control. Macrophages were incubated with IL-10 for 72 h to obtain M2-like macrophages. Simultaneously, IL-10-stimulated macrophages were additionally stimulated with IFN-γ, NPs or IFN-γ-NPs for 72h. **a.** DC (upper panel) and macrophage (lower panel) viability was determined by staining for cell surface Annexin V and Propidium Iodide (PI) followed by flow cytometry analysis. Numbers in gates indicate the percentage of these populations. **b.** Annexin V vs PI plots from gated single cells show the % of populations corresponding to early (Annexin V^+ PI^-) or **c.** necrotic (Annexin V^+ PI^+) cells. **d.** Metabolic activity was evaluated by Rezasurin assay and compared with control cells (unstimulated DCs or unstimulated macrophages). Median is represented by the horizontal line inside the box plots. Data is representative of at least 3 different blood donors. All comparisons were performed using the Friedman test followed by Dunn's multiple comparison test relative to unstimulated DCs or unstimulated macrophages. No significant differences were observed.

3.4. IFN-γ-NPs induce STAT1 phosphorylation

The biological effects of IFN-γ is mainly mediated through the JAK/STAT signaling pathway. Upon IFN-γ binding, STAT1 is phosphorylated in the C-terminus on tyrosine Y701 residues by JAK, resulting in the formation of STAT1 homodimers complexes [56]. To evaluate if the IFN-γ incorporation in Ch/γ-PGA NPs signals through the IFN-γ receptor, the STAT1 phosphorylation was evaluated by western blot analysis in IL-10-treated macrophages stimulated with IFN-γ-NPs or NPs. IFN-γ treatment was used as positive control. STAT1 phosphorylation was not observed in IL-10-stimulated macrophages treated or not with NPs while IFN-γ-NPs induced STAT1 phosphorylation (Fig. 4 a,b), indicating that IFN-γ released from NPs is biologically active.

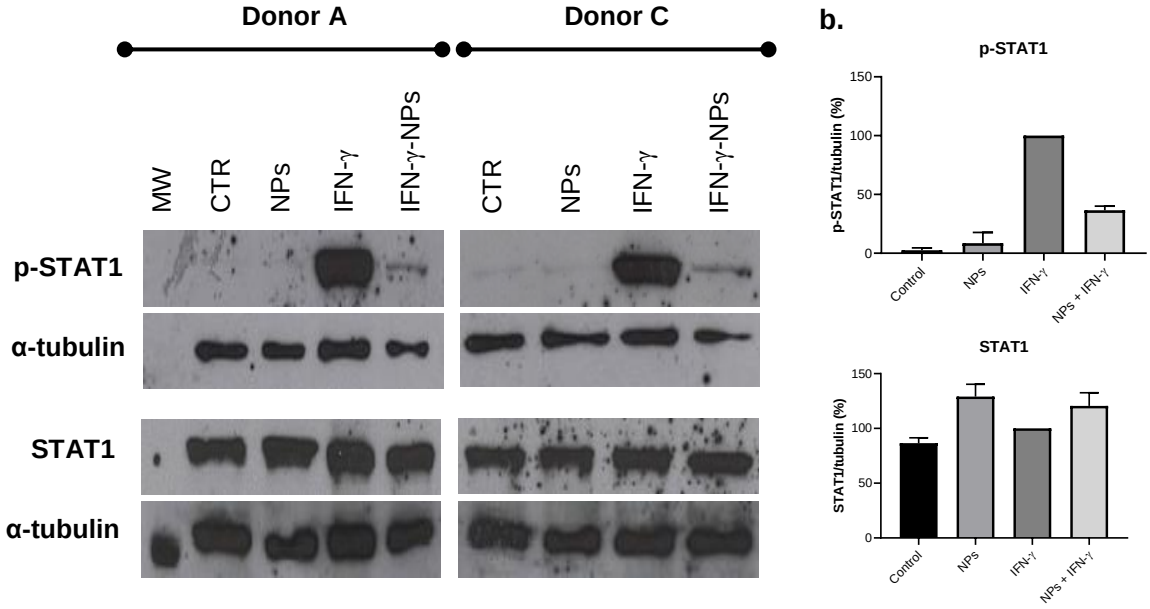

Figure 4. IFN-γ incorporation on Ch/γ-PGA NPs induces STAT Tyr701 phosphorylation. IL-10-treated macrophages were treated with NPs, IFN-γ or IFN-γ-NPs for 1 h. Cellular extracts were assayed by western blots for STAT1 Tyr701 phosphorylation and total STAT1 expression. The results were normalized for α-tubulin expression. **b.** Data were quantified by band densitometry and are expressed as relative percentage to IFN-γ-stimulated macrophages. Phospho-STAT1 and total STAT1 pixel density were first normalized to that for α-tubulin in each sample. Data is representative of 3 donors.

3.5. IFN-γ-NPs affect DCs immunostimulatory profile in a Ch/γ-PGA NPs dependent way

DCs differentiation and activation is frequently impaired at the tumor microenvironment favoring the tumor immune escape [22]. Currently, IFN-γ is being included in pro-inflammatory cocktails to condition monocyte-derived DCs in preclinical and clinical cancer therapy [57]. Here, we experienced that IFN-γ alone is not enough for DCs maturation. We did not observe any alterations in costimulatory molecules expression at DC surface nor on pro-inflammatory and anti-inflammatory cytokines secretion after IFN-γ treatment, consistent with an immature DC phenotype (Fig. 5a,b), Conversely, LPS-treated DCs (maturation control) strongly enhanced both the median fluorescence intensity (MFI) and the percentage of cells that expressed CD40, CD83, CD86 and HLA-DR (Fig. 5a). NPs also increased the expression of these molecules on DCs to levels similar to LPS (Fig. 5a), as previously reported [44]. Regarding to IFN-γ-NPs, treatment enhanced in higher extension the MFI of CD40 and CD86 ($p<0.0014$ and $p<0.0003$, respectively) than NPs treatment ($p<0.0278$ and $p<0.0035$, respectively), comparing to unstimulated DCs. However, IFN-γ-NPs generally did not significantly increase the MFI and the percentage of DCs expressing costimulatory molecules, in comparison to NPs alone. DC activation in response to the distinct treatments was confirmed by cytokine secretion profile. NPs stimulated the production of the pro-inflammatory cytokines IL-6, IL-12/IL-23(p40) and TNF-α, relatively to unstimulated cells, and to similar levels of LPS-treated DCs (Fig. 5b). In addition, IFN-γ-NPs treatment increased IL-6 and TNF-α secretion levels ($p<0,0011$ and $p<0.002$, respectively) further than NPs treatment alone ($p<0.007$ and $p<0.01$, respectively), in comparison to unstimulated DCs. However, IFN-γ-NPs did not significantly enhance the overall cytokine production levels in comparison to NPs-treated DCs. Interestingly, LPS, NPs and IFN-γ-NPs treatments enhanced IL-10 production in comparison to unstimulated or IFN-γ-stimulated cells. Altogether, these results suggest that, at this concentration, IFN-γ incorporation on Ch/γ-PGA NPs does not significantly potentiate the Ch/γ-PGA immunostimulatory properties on DCs.

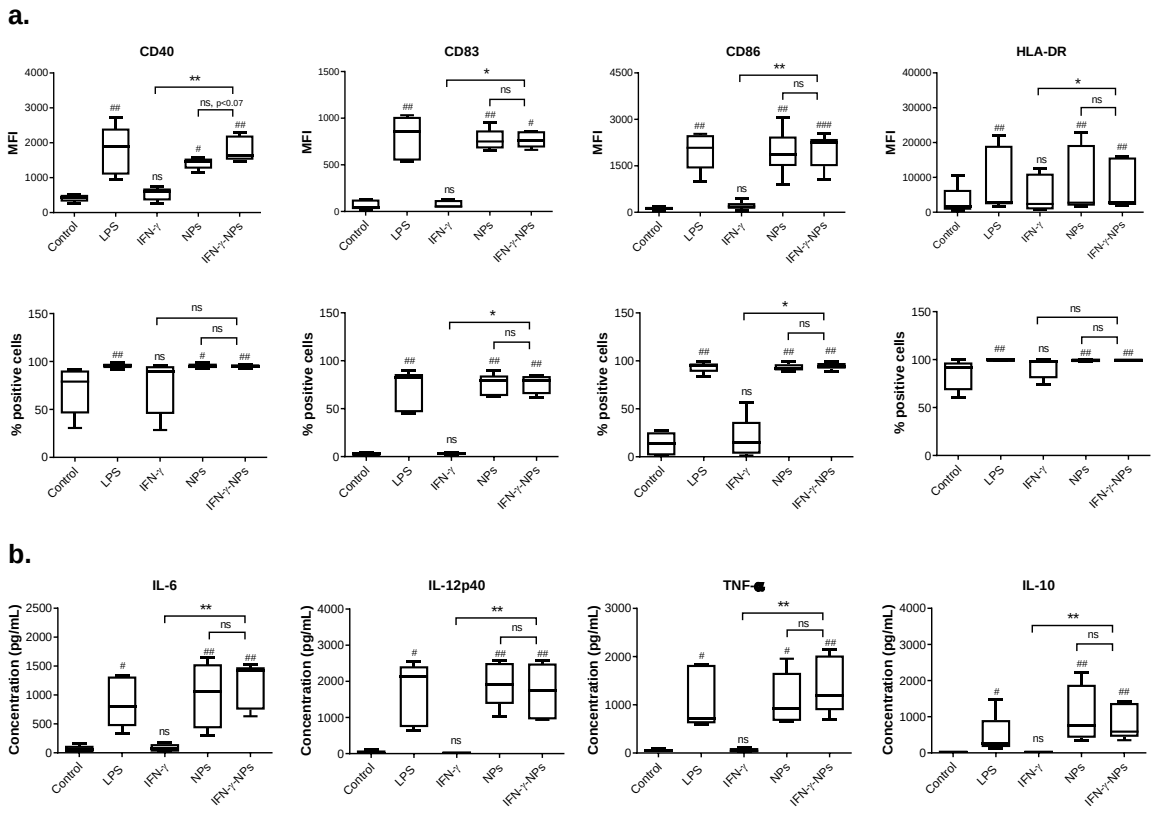

Figure 5. IFN-γ incorporation in Ch/γ-PGA NPs does not seem to potentiate the immunostimulatory profile of DCs induced by Ch/γ-PGA NPs. Differentiated DCs were stimulated with LPS, as positive control, or with IFN-**γ**, NPs or IFN-**γ**-NPs for 48 h. Cells were surface stained for the lineage marker CD11c and the activation/maturation markers CD40, CD83, CD86 and HLA-DR and analyzed by flow cytometry. **a. Top panel:** The median fluorescence intensity (MFI) of CD40, CD83, CD86 and HLA-DR was determined by subtracting the fluorescence intensity of the respective isotype control; **Bottom panel:** percentage of positive cells was determined. Data of at least 6 donors is represented in box plots, where the horizontal line indicates the median. **b.** DC conditioned media were recovered and levels of the pro-inflammatory cytokines IL-6, IL-12/IL-23(p40), TNF-α, and of the anti-inflammatory cytokine IL-10 were determined by ELISA. Data of at least 5 donors is represented in box plots, where the horizontal line indicates the median. In all cases comparisons were performed using the Friedman test followed by Dunn's multiple comparison test ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ relative to unstimulated DCs and $^{*}p<0.05$; $^{**}p<0.01$ relative to IFN-γ-stimulated DCs).

3.6. IFN-γ-NPs slightly impacts macrophage immunostimulatory profile

Previous work from our group demonstrated that IFN-γ release from Ch/γ-PGA PEMs modulated IL-10-treated macrophages (M2-like) towards an immunostimulatory profile [41]. In addition, we have recently reported that Ch/γ-PGA NPs reprogrammed M2-like macrophages towards an immunostimulatory and antitumor profile (M1-like) [44]. In the present work, we asked if the incorporation of IFN-γ on these NPs could potentiate their

effect. First, we investigated the capacity of IL-10-stimulated macrophages to internalize these IFN-γ-NPs. Using previously FITC(ft)-labelled chitosan, the internalization of IFN-γ-NPs was evaluated after 72 h by Imaging Flow Cytometry. After single cells selection, an internalization mask was applied on the brightfield image and the internalization score (ratio between FITC intensity inside the cell and FITC fluorescence intensity of the entire cell) was calculated (Fig. S2a). Representative dot plot and histogram profiles for the ft(IFN-γ-NPs) internalization by human IL-10-treated macrophages are included in Fig. S2b, showing that 67.9 ± 3.7% of the total macrophages internalize ft(IFN-γ-NPs) (ftNPs+ cells). NPs cellular localization (cytoplasmic or membrane) was also determined within ftNPs+ cells, revealing that 98.1 ± 0.7% of the macrophages had higher fluorescence in the cytoplasm, while 1.95 ± 0.7% of the macrophages presented higher fluorescence intensity at the cell membrane, indicating a high internalization efficiency.

In order to evaluate IFN-γ-NPs ability to inhibit M2 polarization, IL-10-treated macrophages were further stimulated with IFN-γ-NPs and their respective controls. The cell surface expression of HLA-DR, CD86 and CD163, M1- and M2-like markers respectively, was evaluated by flow cytometry. IL-10-treated macrophages were included as control. All treatments slightly increased the MFI for the monocyte lineage marker CD14, without reaching statistical significance (Fig. 6a). However, the percentage of CD14-expressing cells remained unaltered. IFN-γ stimulation increased both the MFI and the percentage of HLA-DR and CD86 positive cells (Fig. 6a), as previously described [58]. However, the secretion of pro-inflammatory cytokines IL-6, IL-12/IL-23(p40) and TNF-α was not induced (Fig. 6b). IL-10-macrophages treated with NPs showed no alterations in MFI and percentage of positive cells for HLA-DR and CD86. Conversely, the MFI and the percentage of CD163-expressing cells was significantly decreased (Fig. 6a), which was accompanied by a significant increase in the secretion of the pro-inflammatory cytokines analyzed (IL-6, IL12p40 and TNF-α), in comparison to the control (Fig. 6b). The effects of IFN-γ-NPs were similar to the ones obtained with NPs, with the exception of CD86 MFI, which was significantly enhanced, and the percentage of CD86-expressing cells was slightly increased in comparison to NPs treatment alone ($p<0.07$). The levels of CD163 and the levels of pro-inflammatory cytokines were not affected by IFN-γ-NPs stimulation comparing to NPs stimulation. Interestingly, the IL-10 levels were maintained after the different treatments (Fig. 6b). Altogether, these results suggest that the incorporation of IFN-γ in the NPs slightly strengthens their immunostimulatory effect on macrophages.

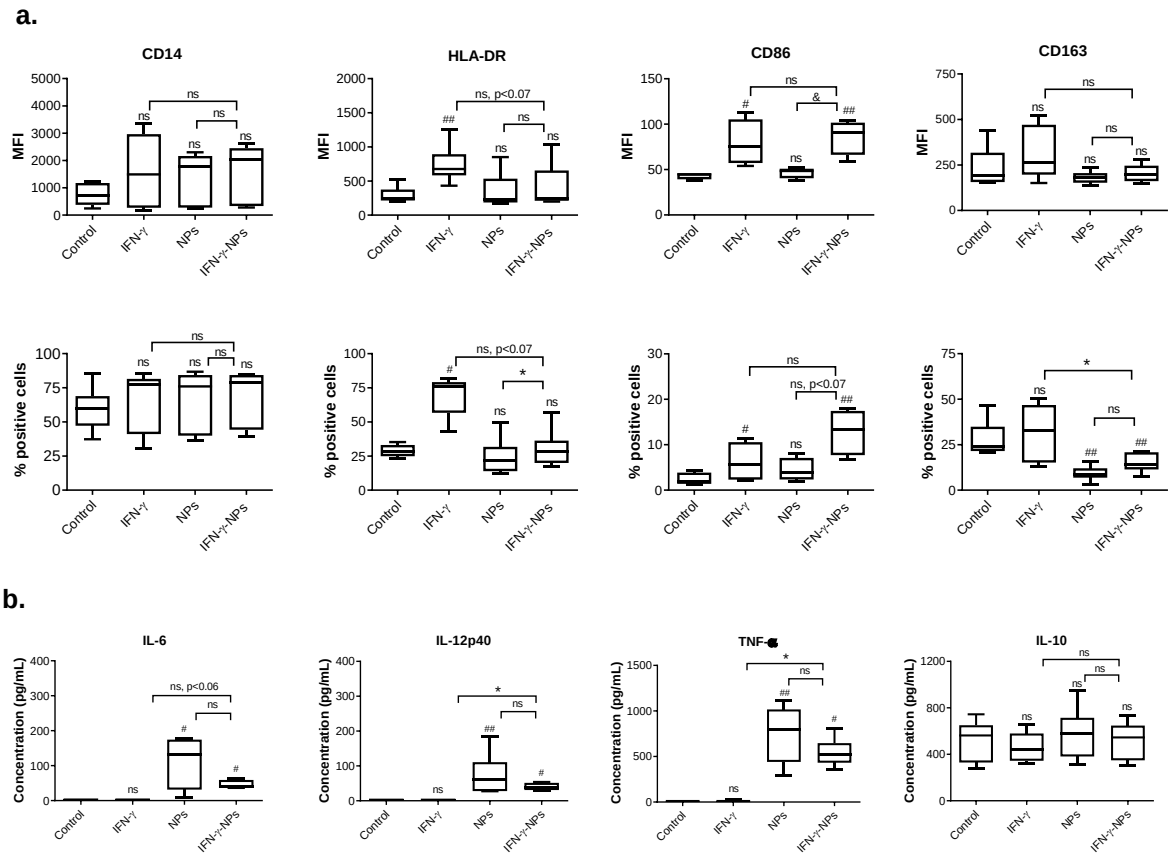

Figure 6. IFN-γ-NPs impacts macrophages immunostimulatory profile. Macrophages were incubated with IL-10 for 72 h to obtain M2-like macrophages. Simultaneously, IL-10-stimulated macrophages were additionally stimulated with IFN-γ, NPs or IFN-γ-NPs for 72h. Then, macrophages were surface stained for the monocyte/macrophage lineage marker CD14, the M1 markers CD86 and HLA-DR, and the M2 marker CD163, and were analyzed by flow cytometry. **a. Top panel:** MFI of CD14, HLA-DR, CD86 and CD163 was determined by subtraction of the fluorescence intensity of the respective isotype control. **Bottom panel:** the percentage of positive cells was determined. Data is representative of at least 4 donors; **b.** Macrophage conditioned media were recovered and levels of the pro-inflammatory cytokines IL-6, IL-12/IL-23(p40), TNF-α and of the anti-inflammatory cytokine IL-10 were determined by ELISA (n=6). Comparisons were performed using the Friedman test followed by Dunn's multiple comparison test ($^{\#}p<0.05$; $^{\#\#}p<0.01$ relative to IL-10-stimulated macrophages, $^{*}p<0.05$ relative to IFN-γ-stimulated macrophages and $^{\&}p<0.05$ relative to NPs-stimulated macrophages). Median is represented by the horizontal line inside the box plots.

3.7. IFN-γ incorporation in Ch/γ-PGA NPs does not potentiate T cell proliferation

Activated antigen presenting cells, as DCs and macrophages, are responsible to induce T cell activation triggering the antitumor immunity [59]. To understand the impact of IFN-γ-NPs on DC and macrophage immune function, DC- and macrophage-induced T cell proliferation was assessed. Therefore, differentiated DCs and macrophages, previously stimulated with IFN-γ, NPs or IFN-γ-NPs, were cultured with CFSE-labeled lymphocytes isolated from a different human blood donor, setting up a mixed lymphocyte reaction. Seven days after co-culture, lymphocytes were harvested and stained with antibodies for CD3, CD4 and CD8 surface markers. CFSE-lymphocytes were also cultured alone, with GM-CSF and IL-4, LPS, IL-10, NPs, IFN-γ or IFN-γ-NPs as experimental controls. Phytohaemagglutinin (PHA) stimulation was used as positive control. As expected, CFSE-lymphocytes alone or treated with GM-CSF and IL-4, LPS, IL-10, IFN-γ, NPs or IFN-γ-NPs did not proliferate while PHA induced T cell proliferation in a dose dependent manner (Fig. S3a,b). Monocyte-derived DCs ($CD11c^+$ cells) were able to induce T cell proliferation (Fig. 7a). LPS-treated DCs, contrarily to IFN-γ-treated ones, significantly increased the percentage of proliferating $CD4^+$ and $CD8^+$ T cells, when compared to unstimulated DCs (Fig. 7a). NPs-stimulated DCs significantly increased the percentage of $CD4^+$-dividing cells while the $CD8^+$ T cell proliferation had a slight increase comparing to unstimulated DCs ($p<0.1$). This effect is mediated by DCs since lymphocytes stimulated with NPs alone did not proliferate (Fig. S3a,b). Nevertheless, IFN-γ-NPs stimulation induced similar levels of $CD4^+$ and $CD8^+$ T cell proliferation in comparison to NPs-treated cells, suggesting that at this concentration the incorporation of IFN-γ in NPs does not potentiate their stimulatory effect regarding DCs-induced T cell proliferation.

Regarding macrophages, we verified that IL-10-stimulated macrophages were able to induce T cell proliferation (Fig. 7b), in agreement with our previous results. Similarly to what happened with IFN-γ-treated DCs, IL-10-treated macrophages incubated with IFN-γ did not increase the percentage of $CD4^+$- and $CD8^+$-dividing cells. Importantly, IL-10-stimulated macrophages treated with NPs significantly increased the $CD8^+$ T cell proliferation ($p<0.004$) while the percentage of CD4-dividing cells was just slightly increased ($p<0.1$), as previously reported. Unexpectedly, the IFN-γ-NPs decreased $CD4^+$ and $CD8^+$ T cell proliferation in comparison to NPs-stimulated macrophages. In summary, these results suggest that at these concentration IFN-γ incorporation on NPs does not potentiate their stimulatory effect regarding IL-10-stimulated macrophages-induced T cell proliferation.

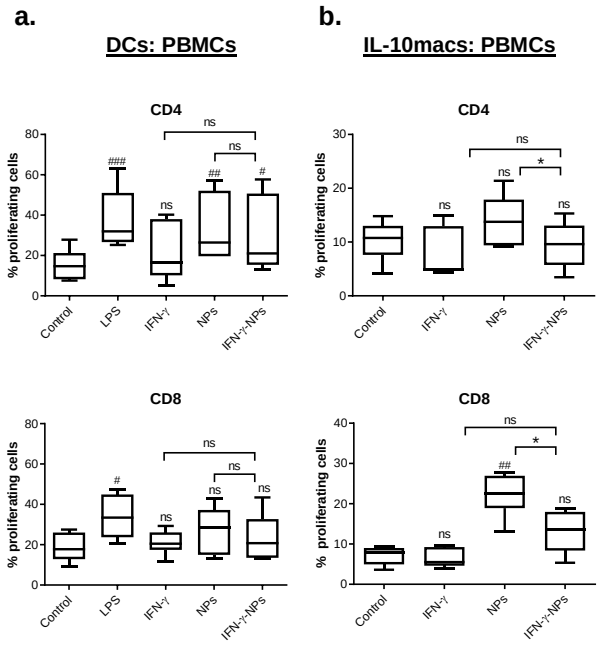

Figure 7. IFN-γ incorporation in Ch/γ-PGA NPs does not potentiate T cell proliferation induced by Ch/γ-PGA NPs. Lymphocytes isolated from a different blood donor labeled with allogeneic carboxyfluorescein succinimidyl ester (CFSE) were added to DCs stimulated with LPS, IFN-γ, NPs or IFN-γ-NPs at a ratio of 1:10 (DCs:lymphocytes) and to IL-10-stimulated macrophage left untreated or treated with IFN-γ, NPs or IFN-γ-NPs at a ratio of 1:8 (macrophages:lymphocytes). After 7 days of co-culture, T cells were surface stained for CD3, CD4 and CD8 and analyzed by flow cytometry. **a. and b.** The percentage of proliferating CD4 and CD8 cells, measured at day 7 of co-culture with DCs and macrophages, were expressed as % of dividing cells in response to the antigenic stimulus. Data is representative of at least 6 different blood donors and median is represented by the horizontal line inside the box plots. Comparisons were performed using the Friedman test followed by Dunn's multiple comparisons test ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ relative to unstimulated DCs or IL-10-stimulated macrophages and * $p<0.05$ relative to NPs-stimulated DCs or macrophages).

3.8. IFN-γ incorporation in Ch/γ-PGA NPs decreases colorectal cancer cell invasion

M2-like macrophages have been described as efficient inducers of cancer cell invasion [18]. Previous work from our group demonstrated that IFN-γ released from PEMs decreased the ability of macrophages to induce cancer cell invasion [41]. To evaluate if the IFN-γ-NPs-induced alterations in DCs and macrophages profile impacted cancer cell invasion, Matrigel invasion assays were performed. Therefore, human colon cancer cells, RKO, were confronted with DCs or IL-10-stimulated macrophages, treated or not with IFN-γ-NPs. LPS-stimulated DCs significantly decreased RKO cell invasion (Fig. 8a) while soluble IFN-γ slightly decreased the stimulation of invasion by DCs ($p<0.1$). NPs stimulation partially

impaired cancer cell invasion mediated by DCs ($p<0.0015$), in agreement with what we previously reported [44]. IFN-γ-NPs-treated DCs were more efficient in reducing cancer cell invasion ($p<0.0009$) although no significant differences were found when compared with NPs-treated DCs (Fig. 8a). Regarding the macrophage-mediated invasion, IFN-γ stimulation slightly decreased cancer cell invasion (Fig. 8b). As expected, NPs treatment significantly decreased the pro-invasive capacity of IL-10-stimulated macrophages ($p<0.01$). Although no statistical differences were found between NPs- and IFN-γ-NPs-treated cells, the latter treatment had a more prominent effect ($p<0.0005$). Altogether, these results suggest that incorporation of IFN-γ potentiates, in some extent, the Ch/γ-PGA NPs ability to impair DCs and macrophage-mediated cancer cell invasion.

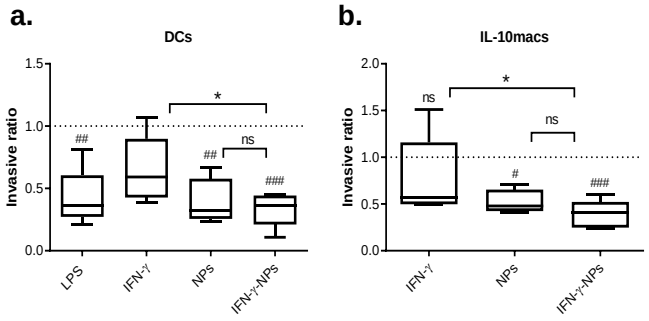

Figure 8. **IFN-γ incorporation in Ch/γ-PGA NPs potentiate their capacity to impair DCs and macrophages–mediated colon cancer cell invasion. a.** DCs treated with LPS, IFN-γ, NPs or IFN-γ-NPs, and **b.** macrophages treated with IL-10, in the presence or not of IFN-γ, NPs or IFN-γ-NPs, were seeded in a well of a 24-well invasion plate. RKO colon cancer cells were seeded inside transwell inserts (with 8 µm-pore size membranes) coated with Matrigel, which were placed above DC and macrophages cultures. After 24 h, insert membranes were removed and the number of invading cells were counterstained with DAPI, and counted under the microscope. Data is representative of at least 5 donors and median is represented by the horizontal line inside the box plots. The results were normalized to unstimulated DCs or to IL-10-stimulated macrophages. Comparisons were performed using the Friedman test followed by Dunn's multiple comparisons test ($^{\#}p<0.05$ ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ relative to unstimulated DCs or IL-10-stimulated macrophages and * $p<0,05$ relative to IFN-γ-stimulated DCs or macrophages).

4. DISCUSSION

In this study, we described a strategy to incorporate IFN-γ within Ch and γ-PGA NPs, a platform with potential as a delivery system, but also with immunomodulatory properties. Based on previous studies, Ch/γ-PGA NPs have been used as suitable delivery systems for bioactive molecules as insulin, anti-inflammatory drugs, namely diclofenac, and antibiotics, specifically amoxicillin [45-48]. Furthermore, our group incorporated IFN-γ into Ch/γ-PGA multilayers (PEMs) which showed biological activity by counteracting macrophage-mediated cancer cell invasion [41]. Despite the promising results, PEMs are 2D structures that cannot be easily applied in clinical settings. Thus, this work prompted the development of an injectable IFN-γ delivery system suitable for *in vivo* applications based on Ch and γ-PGA. These are appealing biomaterials for biomedical applications due to their biodegradability, biocompatibility, nontoxicity and immunostimulatory properties, and thus offer a huge potential for vaccine development [42, 44, 60-64]. We recently reported that Ch/γ-PGA NPs had a prominent effect in inducing macrophages and DCs immunostimulatory phenotype, favoring T cell proliferation and inhibiting CRC cell invasion [44].

The interest in IFN-γ as a target of antitumor immunity appeared around 30 years ago [65]. Although the comprehensive evidences implicating IFN-γ in antitumor immunity, IFN-γ-based therapies had limited success. In fact, several limitations inherent to its molecular properties have been described, including stability problems, as acid degradation, and the tendency to aggregate irreversibly, under mild denaturing conditions, with subsequent loss of biological activity (reviewed in [34, 66]). Additionally, IFN-γ administered intravenously is quickly cleared from the blood, demanding frequent administrations and high cytokine concentrations to trigger an effective response [67], resulting in systemic toxicity and severe adverse effects, namely nausea, fatigue, fever, diarrhea, neurotoxicity and leukopenia [68]. IFN-γ-mediated responses are positively associated with patients' survival in several cancers [69, 70] and some clinical trials present encouraging results [71-73]. Nevertheless, restrictions in IFN-γ-based therapies prompted the development of alternative delivery systems with the purpose of decreasing its toxicity and, simultaneously, achieving better therapeutic outcomes. Several studies have focused on efficient routes for IFN-γ delivery in clinical settings [74-79]. In terms of therapeutic efficacy, an IFN-γ localized delivery has been shown to be more suitable, due to its specific effect at the target site, while simultaneously intensifying the aimed cytotoxic effects and immunological stimulation [35]. Local delivery of IFN-γ was been attempted with liposomes, polymer gels, biodegradable

microspheres, gene therapy, and magnetic or albumin nanoparticles [39, 40, 80-82]. So far, these strategies exposed some limitations namely failure in maintaining IFN-γ stability and/or with biological activity prior to release, an insufficient release rate, an expensive manufacture, and safety issues.

Here, we explored the potential of Ch and γ-PGA NPs as delivery systems for IFN-γ, taking advantage of their immunomodulatory properties, and investigated their ability to reprogram DCs and macrophages towards an immunostimulatory and antitumor phenotype. Ch/γ-PGA NPs are known for their ability in maintaining adequate protein structure and bioactivity, but also due to fact that their assembly process can be performed under mild conditions, without the use of organic solvents. In the present study, IFN-γ (pI=10.27) was combined with Ch/γ-PGA NPs in acidic conditions similarly to what was previously reported [41]. Since IFN-γ is positively charged at the range of pH's in which Ch and γ-PGA spontaneous self-assemble, anionic γ-PGA was dropped to a cationic Ch/IFN-γ solution leading to stable NPs formation by electrostatic interactions. We demonstrate that we could incorporate up to 150 ng/mL of IFN-γ without compromising NPs formation. The release of IFN-γ was evaluated at pH 6, to mimic the more acidic pH present at the tumor microenvironment (TME), and at physiological pH. IFN-γ release was higher at pH 6 than at 7.4, which supports the potential for application of these IFN-γ-NPs within the tumor microenvironment.

Aiming at evaluating the ability of IFN-γ-NPs to modulate DCs and macrophages phenotype *in vitro,* we first investigated the cytotoxicity of these NPs on the distinct antigen presenting cells cultures. At concentrations up to 0.7 mg/mL, IFN-γ-NPs did not impact neither the viability nor the metabolic activity of stimulated DCs and macrophages. DCs activation and maturation was confirmed by surface markers expression and pro-inflammatory cytokines secretion. IFN-γ stimulation of DC maintained the physiological levels of costimulatory molecules and did not induce the secretion of pro-inflammatory cytokines. Instead, Ch/γ-PGA NPs enhanced CD40, CD83, CD86 and HLA-DR expression and induced high secretion of IL-6, IL-12(p40) and TNF-α, as expected [44] while the incorporation of IFN-γ on NPs did not potentiated the acquisition of the immunostimulatory profile already induced by the Ch/γ-PGA NPs. Functionally, DCs treated with NPs were efficient in activating allogeneic T cells, significantly enhancing $CD4^+$ T cell response. As expected, IFN-γ-NPs did not further potentiate T cell proliferation, as observed in Ch/γ-PGA NPs-treated DCs, since IFN-γ-NPs did not enhance neither the expression of costimulatory molecules nor the ability of DCs to secrete pro-inflammatory cytokines. Several reports have studied the effect

of IFN-γ on DCs maturation and activation. Frasca *et al.,* showed that monocyte-derived DCs (MDDCs) stimulated with IFN-γ presented physiological levels of co-stimulatory molecules, except for stimulation with higher concentrations (500 U/mL). IFN-γ itself did not promote relevant CCR7-driven migration or activated Th-1 cell phenotype. However, co-administration with maturation signals, as CD40 ligand and LPS, led to an up-regulated of IL-27 and IL-12p70 production, CCR7-driven migration, and activated Th-1 cell recruitment [83], which suggest that IFN-γ treatment of autologous MDDCs used in cancer therapy could benefit from a combination with maturation signals as CD40 ligand or TLR ligands. Indeed, a pro-inflammatory cocktail combining TNF-α, IL-1β, poly:IC, IFN-α and IFN-γ has been used in clinical trials to induce the maturation and activation of MDDCs [84, 85]. The administration of DCs maturated with this cocktail, and loaded with neoantigen peptides, in patients with high grade glioma proved to be safe and immunogenic, and resulted in progression-free status in 9/22 patients for at least 12 months [86]. Our strategy followed this rational, since IFN-γ was combined with a maturation stimulus - the Ch/γ-PGA NPs, still the cellular effects observed were mainly Ch/γ-PGA NPs-mediated.

In parallel, the effect of IFN-γ-NPs on macrophage profile was studied by analyzing several phenotypic and functional parameters described for M2-polarizing macrophages. Previous studies have demonstrated that IFN-γ stimulation (50 ng/mL, 24 h) of peripheral blood monocytes after LPS activation, in the presence of ovarian ascites, switched monocyte differentiation from M2-like to M1-like macrophages, characterized by an increase of IL-12 secretion and CD86 costimulatory molecule expression [31]. Here, we verified that IFN-γ stimulation induced expression of HLA-DR and CD86 by IL-10-treated macrophages but did not impact on the CD163 expression neither on the secretion of pro-inflammatory cytokines, suggesting that the M1-like profile (enriched on HLA-DR, CD86 expression and high pro-inflammatory cytokines secretion, and low CD163 expression and IL-10 secretion) was not completely achieved. Others also reported that IFN-γ alone did not induce a complete M1-like profile but the combination with TLR agonists induced tumoricidal activity and production of pro-inflammatory mediators [87]. Nevertheless, Ch/γ-PGA NPs were able to inhibit M2 macrophage polarization, by decreasing the expression of CD163 and promoting pro-inflammatory cytokines secretion, as previously reported [44]. In addition, IFN-γ-NPs significantly increased CD86 expression by IL-10-stimulated macrophages but the impact on HLA-DR, CD163 expression and on pro-inflammatory cytokines production was negligible in comparison to Ch/γ-PGA NPs, suggesting that IFN-γ incorporation on NPs slightly impact the Ch/γ-PGA NPs effects. These results can be related with a lower STAT1

phosphorylation induced by IFN-γ-NPs comparing to IFN-γ-stimulated macrophages. Indeed, the synergistic effect of TLR agonists and IFN-γ stimulation is mediated through a STAT1-dependent mechanism [88]. NPs did not induce STAT1 activation until 1 h after stimulation which is in accordance with delayed STAT1 activation by Ch microparticles [89]. Functionally, IFN-γ-NPs-stimulated macrophages decreased $CD4^+$ and $CD8^+$ T cell proliferation which is in accordance with the lower secretion of pro-inflammatory cytokines by IFN-γ-NPs-stimulated macrophages. These results can constitute an IFN-γ mechanism to repress the inflammatory program induced by Ch/γ-PGA NPs [90].

Considering the protumor role of M2-like macrophages [18], we evaluated the ability of IFN-γ-NPs to counteract the pro-invasive ability of IL-10-stimulated macrophages on RKO cancer cell invasion. As expected, Ch/γ-PGA NPs significantly reduced DCs and macrophage-mediated cancer cell invasion, likely as consequence of the shift of their immune profile [44], while IFN-γ stimulation only slightly reduced cell invasion. Interestingly, IFN-γ-NPs decreased in higher extension the pro-invasive ability of DCs and IL-10-treated macrophages, possibly through the synergistic effect of IFN-γ and Ch/γ-NPs to induce antitumor M1 phenotype, suggesting that although without a striking shift on the immunomodulatory profile, these IFN-γ-NPs may be improved in the near future and exploited as a tool to impair cancer cell invasion.

Although most of the cellular events observed were Ch/γ-PGA NPs mediated, IFN-γ incorporation assumed a role in the expression of some co-stimulatory molecules, as CD40 on DCs and CD86 on macrophages, and in impairing DCs and macrophages-mediated cancer cell invasion. Considering what is reported, we expected a higher impact of IFN-γ-NPs on macrophage and DCs polarization. Probably, with higher amounts of incorporated IFN-γ, STAT1 activation could be increased and potentiate the immunoregulatory role of IFN-γ. Still, released IFN-γ decreased inflammatory cytokines production by macrophages, followed by a decrease in T cell proliferation, and this should be carefully addressed in the future.

Overall, our results demonstrated that Ch/γ-PGA NPs can be an adequate delivery system for IFN-γ, retaining its biological activity while potentiating some pro-inflammatory features and counteracting the pro-invasive role of DCs and macrophages. This strategy constitutes a proof-of-concept that we can use Ch/γ-PGA NPs for IFN-γ delivery and the combination treatment with IFN-γ and Ch/γ-PGA NPs may offer new avenues for APCs-based cancer immunotherapy.

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Conflict of Interests

The authors declare no conflict of interests.

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Supplementary figures

Supplementary Figure S1

a.

Sample	Molar Ratio Ch:IFNγ:γ-PGA	[IFN-γ] ng/ml	Particle size (nm)	Polydispersity	Zeta potential (mV)
Ch/γ-PGA	1:0:1.5	0	295.0 ± 12.7	0.28 ± 0.01	18.1 ± 0.7
Ch/IFN-γ/γ-PGA	1:0.1:1.5	43	301.7 ± 4.6	0.28 ± 0.01	16.7 ± 1.9
	1:0.25:1.5	107	301.2 ± 9.1	0.27 ±0.01	18.3 ± 0.4
	1:0.35:1.5	150	315.6 ± 8.9	0.28 ± 0.01	18.6 ± 1.4
	1:0.5:1.5	214	319.6 ± 26.6	0.43 ± 0.05	15.5 ± 1.1
	1:0.7:1.5	300	855.7 ± 47.5	0.66 ± 0.03	-5.1 ± 0.5

b.

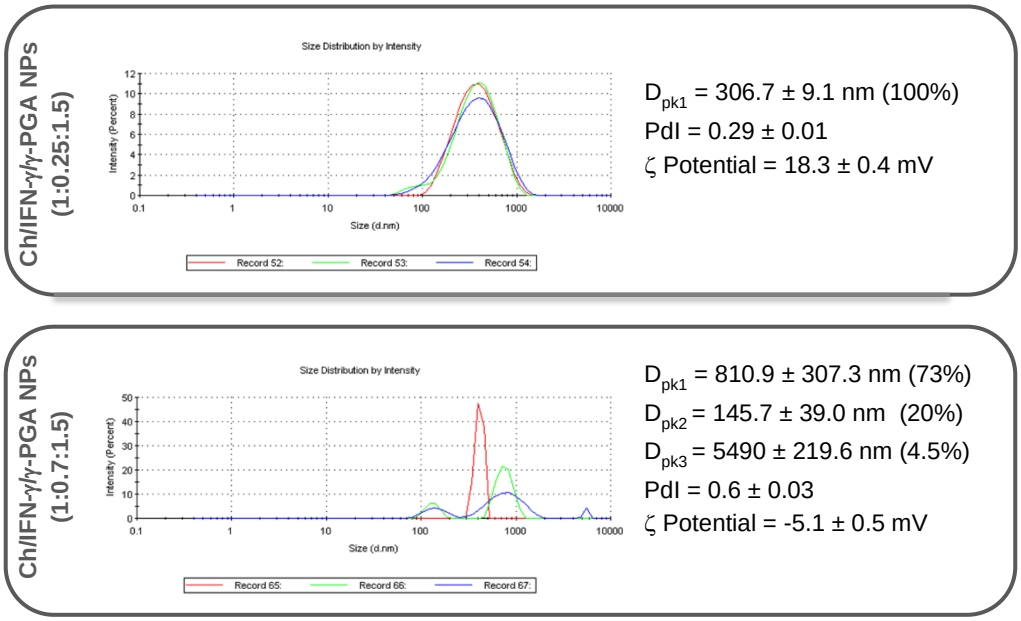

Figure S1 – **Characterization of IFN-γ-NPs by DLS**. **a.** IFN-γ-NPs were prepared at different molar ratios of IFN-γ (0.1, 0.25, 0.35, 0.5 and 0.7), maintaining Ch and γ-PGA molar ratios constant, 1 and 1.5, respectively. After preparation, their size (d, nm), polydispersity index (PdI) and ζ potential (ζ pot., mV) measurements were determined by dynamic light scattering (DLS). **b.** Particle size distribution plots from a monodisperse (1:0.25:1.5) and a multidisperse preparation (1:0.7:1.5) were obtained by intensity of the scattered light, using the same equipment.

Supplementary Figure S2

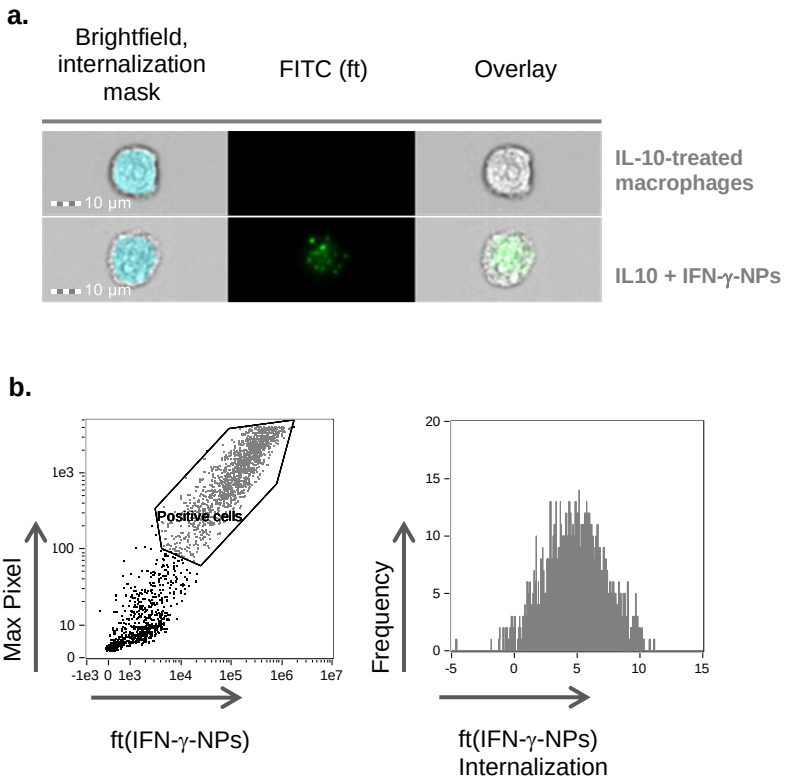

Figure S2 – **IFN-γ-NPs are internalized by macrophages**. Human macrophages were stimulated with IL-10 alone or in combination with IFN-γ-NPs for 72 h. Cells were recovered and fixed for Imaging Flow Cytometry. **a.** The percentage of NPs internalization was determined after application of an internalization mask on the cell population of positive cells for FITC(ft) ft(IFN-γ-NPs). Each cell is represented by a row of three images acquired simultaneously in flow, from left to right: brightfield with a cytoplasm mask represented in blue, FITC fluorescence (green) for the ft(IFN-γ-NPs), merged image (scale bars, 10 µm). **b.** Representative dot plot and histogram profile for the ft(IFN-γ-NPs) internalization by macrophages (lower panel) by Imaging Flow Cytometry. Data is representative of 2 donors.

Supplementary Figure S3

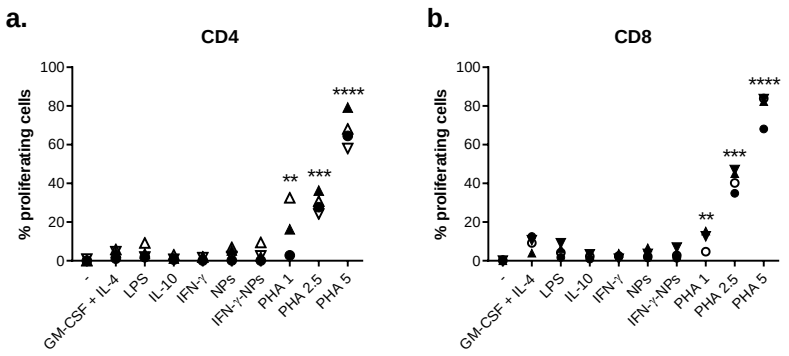

Figure S3- Mixed lymphocyte reaction controls. Lymphocytes isolated from a blood donor different from those used for monocyte isolation were labeled with allogeneic CFSE and cultured alone (-) or with DCs differentiating cytokines (GM-CSF and IL-4), LPS, IL-10, IFN-γ, NPs, IFN-γ-NPs or PHA as experimental controls. After 7 days of co-culture, cells were surface stained for CD3, CD4 and CD8 followed by flow cytometry analysis. **a**. The percentage of $CD4^+$ and **b**. $CD8^+$ dividing cells were determined. Comparisons were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test (**p<0.01; ***p<0.001; ****p<0.0001).

CHAPTER VIII

General Discussion and Future Perspectives

During the past few decades the advances in cancer biology research have contributed to improve our knowledge regarding the tumorigenesis process, mainly on the determinant factors for cancer initiation and progress. Currently, it is known that tumors are complex tissues composed by multiple cell types and extracellular matrix components, resulted from a combination of genetic and environmental factors growing in a permissive environment, which determines tumor outcome and response to therapy [1-3]. Accumulating evidences over the last years, from mouse models and human patients with cancer, demonstrated the relevance of the immune system in the recognition and eradication of transformant malignant cells [4, 5]. However, the discovery of tumor cells ability to subvert and evade the immune system was an essential hallmark for understanding tumor progression and therapeutic failure [6, 7]. The recent clinical success of immune checkpoint inhibitors and T cell therapy by overcoming or relieving tumor-induced immunosuppression, enabling immune-mediated tumor clearance, improved disease outcome [8-10]. Nevertheless, several challenges have arose, including the need to identify biomarkers to achieve better patients stratification and to predict patient response, treatment efficacy, and the development of resistance [11]. Several preclinical studies based on immune reprogramming have been explored as effective strategies for treating cancer [12, 13]. Accordingly, this PhD thesis aimed to develop an immunomodulatory system, based on the immunostimulatory properties of chitosan (Ch) and of poly(γ-glutamic acid) (γ-PGA), and to clarify its impact on antigen presenting cells (APCs) polarization, its ability to induce an efficient immune response, and to impair APCs-mediated cancer cell invasion. Furthermore, we proposed to test the adjuvanticity of this strategy with classical anticancer therapies, namely radiotherapy, and with the immunomodulatory IFN-γ, in an attempt to potentiate its adjuvanticity for anticancer therapies. We also tested the potential of our strategy to be used as carriers for IFN-γ.

Regarding the first aim, described in the chapter IV, we decided to explore the potential of two well described biomaterials, namely Ch and γ-PGA. They are attractive adjuvants and their biodegradability, biocompatibility and non-toxicity offer a huge potential for cancer treatment. Ch is a polysaccharide, product of chitin deacetylation, which main sources are the fungal cell walls and the shell of crustaceans. Ch has been associated with antimicrobial, antioxidant and antitumoral activities and it was approved by FDA for dietary use and tissue engineering applications [14]. Regarding its antitumoral properties, Ch derivatives have been described to delay *in vitro* cell proliferation of specific tumor cell lines [15, 16] and, to delay tumor progression and metastasis burden of Lewis lung carcinoma (LLC)-bearing mice [17]. Importantly, the adjuvant potential of Ch has been explored despite the

underlying molecular mechanisms are not fully understood. In 2008, Li and colleagues demonstrated that Ch induces inflammasome activation in a NLRP3-dependent fashion [18, 19] and Bueter *et al.* showed that Ch activates NLRP3 inflammasome by a phagocytosis-dependent mechanism [20]. Additionally, Mori and co-workers reported that Ch provides an alternative to alum, which is currently used in vaccines, as it does not inhibit IL-12 production and, in combination with CpG, is a potent inducer of NLRP3 inflammasome-dependent Th1 and Th17 responses [21]. In agreement, Young *et al.* evidenced that DCs coated in a Ch substrate had enhanced functional activation, and that tumor bearing animals vaccinated with DCs, harvested from Ch substrate, increased cytotoxic T lymphocyte (CTL) response, and significantly decreased the tumor growth than those vaccinated with DCs harvested from TCPS substrates [22]. In opposite, others showed that DCs, after differentiation in Ch films, acquire a pro-inflammatory profile while macrophages acquire an anti-inflammatory one, without impact on T cell activation [23]. Other studies have implicated Toll-like receptor 4 (TLR4) in Ch recognition, but the downstream mediators were not analyzed. Villiers and co-workers demonstrated that DCs activation was partially mediated by TLR4 activation, but no pro-inflammatory cytokines were detected, failing priming of T cells [24]. Zhang *et al.* suggested that TLR4 mediated the immunostimulatory effects of Ch oligosaccharides [25]. Most recently, an interesting study evidenced that Ch activates DCs through type I IFN production and promotes Th1 response by engaging the DNA sensor cGAS-STING pathway [26]. This study suggests that Ch induces mitochondrial stress, followed by mitochondrial DNA release and cGAS-STING engagement instead of acting as danger-associated molecular pattern (DAMP). Still, the crosstalk between this pathway and the inflamasome-IL-1β pathway remains to be elucidated. The diverse immune profiles observed upon Ch stimulation is likely a result of different natural sources, molecular weight, manufacturing processes, formulation (solutions, powders, gels and particles), deacetylation degree and/or purity level, which highlights that the standardization is necessary to enable preclinical and clinical advances [27]. Nevertheless, Ch nanoparticles (NPs) have also been extensively explored for vaccine and drug delivery [28-30]. In agreement, Ch NPs coated with surface-decorated mannose were considered efficient vehicles for cancer vaccine delivery. When combined with BL6 tumor lysates, they had therapeutic efficacy in B16 tumor bearing mice, associated with an enhanced CTL response [29]. Ch-lactate NPs loaded with CD73 specific siRNA potentiate the antitumor effect of a DC vaccine in 4T1 breast cancer bearing mice [28]. The combination of folate-modified Ch NPs containing the IP-10 gene with $CD8^+CD28^+$ CTLs therapy decreased B16 tumor progression and increased mice survival [30] Additionally, the combination of these NPs

with DC/irradiated tumor cells fusion vaccine synergized to decrease hepatocellular carcinoma in mice by decreasing the granulocytic tumor infiltrates [31]. Altogether, these studies point out Ch as an appealing adjuvant for the formulations of cancer vaccines.

On its turn, γ-PGA is a poly-aminoacid obtained by microbial production using one of several strains of *Bacillus* [32]. We obtained from *Bacillus subtilis natto* and was characterized by a low molecular weight (10–50 kDa) and high purity grade (99 %) [33]. Throughout the years, γ-PGA has been explored for applications in the biomedical field, as scaffolds for tissue regeneration, nanocarriers for drug and antigens delivery, biological adhesives, vaccines, as well as in the food, cosmetics and wastewater industries [34]. γ-PGA NPs have been explored for their potential as adjuvants for vaccines production. Uto and co-workers showed that γ-PGA NPs were internalized by DCs and localized inside lysosomal compartments. Importantly, γ-PGA NPs enhanced bone marrow-derived DCs costimulatory molecules expression, cytokines production, T cell stimulatory capacity and involved MyD88-mediated NF-κB signaling pathway. [35]. They also showed the potential of γ-PGA NPs to carry human immunodeficiency virus type 1 gp120 and elicit a stronger antigen-specific CTL response comparing to antigen alone [36], highlighting the potential use as effective adjuvants for vaccines. This potential of γ-PGA NPs was posteriorly explored in murine spleen DCs by demonstrating the ability of γ-PGA NPs to induce long-lasting cellular and humoral immunity [37]. Hamasaki *et al.* showed that the gene expression profile of murine DCs stimulated with γ-PGA NPs was quite similar to that of LPS-treated DCs. In opposite, unarticulated γ-PGA could scarcely impact DCs gene expression, suggesting that the NPs formulation of γ-PGA is required to exert its biological activity [38]. Interestingly, the size of γ-PGA NPs also impacted their cellular uptake and DCs maturation [39]. Additionally, they showed that γ-PGA NPs modulate the expression of TLR signaling-related genes, namely triggered by LPS, zymosan and CpG but not by polyI:C, and evidenced that these NPs were more effective to induce an antigen-specific CTL response than conventional adjuvants, as alum [38, 40]. Later studies showed that γ-PGA NPs induce potent innate and adaptive immune responses through the activation of Toll-like receptor 4 (TLR4) and MyD88 signaling pathways in macrophages and DCs. These evidences suggest that γ-PGA NPs besides its antigen-carrying capacity can effectively act as an adjuvant through the recognition of the first-line host-sensor system [41]. The combination of γ-PGA NPs with CpG synergistically activated macrophages and potentiated antigen-specific T cell response likely due to the multiple TLR activation [42]. The combination with anti-CD40 improved the stimulatory capacity of the CD40 agonist and induced strong synergistic proliferative effects in B cells [43]. γ-PGA NPs combined with EphA2, a tumor-associated

antigen, resulted in the generation of EphA2-specific CTL response and provided a degree of anti-MC38 liver tumor protection, more than that observed for immunization with the mixture of EphA2-derived peptide and complete Freund's adjuvant [44]. These γ-PGA NPs have been demonstrated to be efficient in tumor antigen delivery for APCs in cancer [45], and an intracellular mechanism of efficient cross-presentation induced by OVA-loaded γ-PGA NPs was demonstrated similar to the classical MHC class I presentation pathway for endogenous antigens [46]. Additionally, the ability of γ-PGA to spontaneously self-assemble with Ch allows its application without the need of chemical cross-linking and, offers also opportunities to develop small vehicles for controlled drug release, while provide a great adjuvant potential to other therapeutic strategies. Overall, these studies highlighted the potential of Ch and γ-PGA to be included in therapeutic strategies to further improve both cellular and humoral immune responses.

Considering these properties, we decided to explore the combination of Ch and γ-PGA under nanoparticles formulation. We suspect that several immune pathways are triggered by each of these components, potentiating the observed immune response. It would be certainly interesting to explore, in the near future, the impact of Ch/γ-PGA NPs in the activation of myeloid cells, in comparison with NPs of Ch or of γ-PGA alone, namely on the signaling pathways involved. Importantly, the APCs activation profile, in particular the panel of pro-inflammatory cytokines, induced by Ch/γ-PGA NPs stimulation was closer to the one promoted by γ-PGA NPs than by Ch NPs. Nevertheless, IL-1β and T helper 1 response induction can be induced by Ch stimulation, as previously reported. In addition to DC maturation, the reprograming of primed IL-10-macrophages towards an immunostimulatory profile upon Ch/γ-PGA NPs was a new achievement. Notably, the effects of Ch/γ-PGA NPs on macrophage and DCs profile induced distinct T cell polarization status. While DCs maturation induced CD4 T cell response, macrophages repolarization induced CD8/CTL response. This differential T cell activation can be possibly explained by the impact of Ch/γ-PGA NPs on HLA-DR expression on DCs, which favors antigen presentation to CD4 T cells, while on macrophages MHC class II molecules were not implicated. Considering that the cytotoxic CD8 T cell activation is not mediated by MHC class II but, instead, by MHC class I molecules [47], it would be important to investigate the expression of these molecules on macrophages confronted with Ch/γ-PGA NPs.

IL-10-stimulated macrophages are well described to be associated with pro-tumorigenic activities, such as tumor cell invasion [48-50]. We proved that Ch/γ-PGA NPs were able to decrease the ability of macrophages and DCs to induce tumor cell invasion, but the soluble factors involved in this process were not identified yet. Nevertheless, our group

demonstrated that proteolysis is essential for macrophage-mediated cancer cell invasion [49], and that CCL18 produced by macrophages induce tumor cell invasion [51]. It would be now interesting to perform a molecular screening of the conditioned medium from invasion assays to explore soluble factors possibly affected by Ch/γ-PGA NPs macrophage stimulation, as CCL18 and MMPs. Overall, since Ch/γ-PGA NPs reprogram immune cells towards an immunostimulatory and anti-invasive profile while increasing T cell-mediated immunity, we believe that our results support the need of Ch/γ-PGA NPs to be tested as therapeutic adjuvants in preclinical studies.

Therefore, we decided to explore the adjuvant potential of Ch/γ-PGA NPs to radiotherapy (RT) treatment, using an orthotopic breast tumor model, described in the chapter V. We opted to combine Ch/γ-PGA NPs with RT, since this is one of the most commonly used cancer treatment modalities, and to which approximately 50% of all cancer patients would reach [52]. Although RT is the most-effective cytotoxic therapy available for the treatment of localized solid cancers, many patients have local tumor recurrences following radiotherapy regimens, likely promoted by the recruitment of immunosuppressive players. In fact, it is known that RT while inducing tumor cell death also induces the recruitment of immunosuppressive myeloid cells possibly to protect irradiated tissue from the destruction caused by the profound inflammatory response induced by radiotherapy. In addition, such recruitment dampens the antitumor immune response triggered by the apoptotic tumor cells [53, 54]. In fact, the accumulation of myeloid cells ($CD11b^+$), including MDSCs and TAMs, have been associated with tumor progression and metastasis [50, 55, 56], highlighting the importance of the modulation of these cells at the TME to improve therapy response. Our team recently explored the effect of clinically relevant ionizing radiation doses (5*2 Gy) on human macrophage function and on macrophage-cancer cells crosstalk. It was demonstrated that besides inducing DNA damage, irradiated macrophages remain viable and metabolically active, most probably through the activation of the pro-survival NF-kB signaling pathway. In addition, ionizing radiation drives macrophages towards a more pro-inflammatory phenotype, preserving still their ability to induce cancer cell invasion and angiogenesis [57]. Considering the infiltration of these cell types in the post-radiotherapy microenvironment, there is an opportunity to target these cells following RT and via combination with other treatment modalities to restore T cell-mediated antitumor immune effects and improve the clinical response.

In this study, the 4T1 orthotopic tumor model, a poor immunogenic and highly malignant breast tumor model, characterized to be infiltrated by a high percentage of immunosuppressive myeloid cells, mainly granulocytes ($CD11b^+$ $Ly6G^+$) was used. We

demonstrated that the treatment of 4T1-tumor bearing mice with RT followed by subcutaneous injection of Ch/γ-PGA NPs potentiates Th1 response and impaired the establishment of an immunosuppressive systemic environment, promoting a strong antitumor effect and improving the control of tumor cell spread, in comparison to RT alone and Ch/γ-PGA NPs alone. Although the impact on myeloid cells is still being analyzed, our study serves as a proof-of-concept for future clinical translation. Besides Ch/γ-PGA NPs immunostimulatory properties, it would be interesting to incorporate specific 4T1 tumor antigens, to evaluate the potential of these NPs to be used as part of therapeutic anticancer vaccines.

Irradiation has been recently described to upregulate the expression of PD-L1 on tumor cells in response to DNA damage, which in turn blocks the T and NK cells-mediated activities [58]. This evidence suggests that the blocking of immune checkpoint inhibitors could be a promising strategy to improve the therapeutic effect of radiotherapy, by improving T and NK cells immune response. Preclinical studies evidenced that anti-PD-L1 synergizes with irradiation to induce antitumor immunity [59] and that acquired resistance to fractionated radiotherapy can be overcome by PD-L1 blockade [60]. These promising results from preclinical models encouraged the advances to clinical trials. Currently, more than 100 clinical trials are exploring this combinatorial treatment, but the clinical benefits have been modest in patients with solid tumors [61, 62] likely due to the pre-existent immunosuppressive environment or to the inability to respond to IFN-γ stimulation [63, 64]. Notably, the combinatorial treatment used in this thesis (RT+NPs) generally reduced the expression of cytokines and chemokines with immunosuppressive activities and improved the IFN-γ expression by splenic T cells. Considering these results, we would like to address, in the future, the impact of our therapeutic strategy on the immune checkpoint axis PD-1/PD-L1/PD-L2 on both tumor and immune cells. Additionally, the impact of the combinatory therapy (RT+NPs) on TGF-β expression at the tumor site could be interesting, since this molecule has been described to be a regulator of tumor immunity after irradiation. Vanpouille-Box and colleagues showed that anti-TGF-β combined with radiation therapy elicit effective CD8 T cell response and reduced primary tumors and non-irradiated lung metastasis. However, these animals presented rapid recurrence after up-regulation of the immune checkpoint inhibitors PD-L1 and PD-L2 expression, on tumor and myeloid cells. The combination with anti-PD-1 antibodies improved the survival achieved with the radiation and TGF-β blockade [65]. These studies reinforce the importance of combinatorial treatments to achieve an optimal and more sustained response against cancer.

The effects of ionizing radiation on tumors depend on the dose per fraction applied, the number of fractions and the total dose, and from the interplay between these three variables in a specific tumor microenvironment. To minimize collateral injury to the adjacent normal tissue, the radiotherapeutic regimen most frequently used is of multiple fractions of 2 Gy, and of five fractions per week. In our study, considering the logistic constrains of irradiating animals during an entire week (5*2 Gy), we decided to divide the desired total irradiation dose (10 Gy) into two fractions of 5 Gy. To date, the impact of dose and fractionation on the immunostimulatory potential of radiotherapy has not been sufficiently investigated [66, 67]. Recent data demonstrated that DCs recruitment to tumor site and activation of CD8 T cells is highly dependent on RT dose and fractionation through a mechanism that involves the accumulation of DNA double-strands in the cytoplasm of cancer cells and, which synergizes with anti-PD-1 or anti-CTLA-4 therapy [68]. Authors demonstrated that in contrast to a fractionation scheme (3*8Gy), a high ionizing radiation administration scheme (20Gy) induces the expression of the DNA nuclease Trex1, which instead degrades the double strand DNA breaks released upon radiation in the cell cytoplasm. Such degradation impairs the action of the DNA sensor cGAS and of its downstream effector STING, which would induce through a NF-kB and IFN-β mediated pathway, a pro-inflammatory CD8 immune response [68]. To complete our study, it would be interesting to explore if total dose hypofractionation, as 5*2Gy, which more closely mimics one week of a cancer patient treatment, would improve the outcome obtained, and if the concurrent Ch/γ-PGA NPs administration, starting on the day or during fractionated RT, would be better than starting after RT completion, as it was demonstrated with immune checkpoint blockade treatments [60]. Investigating the effect of both 2*5 Gy or 5*2 Gy on cancer cell MHC class I, PD-1 or PD-L1 expression, as well as on the recruitment and activation of specific CD4 or CD8 T cells populations will contribute at understanding how ionizing radiation fractionation impacts the immune response.

Having demonstrated the adjuvanticity of Ch/γ-PGA NPs to ionizing radiation, we decided to explore their potential to be combined with IFN-γ administration, as described in chapter VI. Despite the dual role of IFN-γ in cancer [69], it is consensual that this cytokine is critical to an efficient antitumor immunity. The recent success in immunotherapy field, namely through the activation of antitumor immunity with immune-checkpoint inhibitors or through autologous CAR/TCR lymphocytes adoptive transfer, has been associated with IFN-γ-dependent antitumor actions [70-72]. Recently, it was demonstrated through a genetic screening of melanoma cells that IFN-γ have a crucial role in the responsiveness to immunotherapies [73, 74]. In addition, the analysis of tumors from relapsed or non-

responders patients to immune checkpoint inhibitors evidenced genomic alterations resulting in IFN-γ signaling-related genes loss (*IFNGR1, IRF1, JAK1, JAK2, IFNGR2)* and amplification of IFN-γ suppressor genes (*SOCS1, PIAS4*) [64, 75-77]. Altogether, these results evidence that tumor IFN-γ responsiveness is critical for antitumor immunity and response to immunotherapy.

In our study, we demonstrated *in vitro* that Ch/γ-PGA NPs and IFN-γ synergize to induce an immunostimulatory and tumoricidal M1 profile. The translation for an *in vivo* setting showed us that the combination of Ch/γ-PGA NPs with soluble IFN-γ administration led to an increase on antitumor immunity, with reduced primary tumor burden and lung metastasis formation, likely associated to decreased $CD11b^+$ myeloid cells and increased T cell response. Notably, while tumor progression was delayed upon single treatments (NPs or IFN-γ), the combinatorial treatment (NPs+IFN-γ) consistently blocked primary tumor growth. This outcome is likely a result from the combination of the immunostimulatory properties of the Ch/γ-PGA NPs which synergize with the immunomodulatory and tumoricidal functions of IFN-γ. It would be interesting to characterize the immune response immediately after treatment being stopped, to understand if the antitumor effects obtained are mainly mediated by early or later alterations in the immune cells profile. We opted to post-pone the end of the experiment to unveil if the tumor growth blockade induced by combinatorial treatment would be maintained in the absence of treatment. However, this was not verified, demonstrating that the inhibitory effect achieved is not sustained after treatment completion, probably given the plasticity of the immune cells to microenvironment stimuli. In this sense, it would be interesting to combine this NPs+IFN-γ strategy with 4T1 tumor antigens to elicit an antigen-specific T cell and memory response. Additionally, it would be interesting to evaluate if the phenotype that we observed was related with the decrease of myeloid cells, by evaluating the impact of their depletion. Since IFN-γ has been recently described to upregulate PD-L1 expression [78], it would be interesting to evaluate the impact of our therapeutic strategy on its expression on tumor and immune cells.

Most of preclinical studies showed the impact of IFN-γ on antitumor immunity but its potential for anticancer therapy is not well understood. Several clinical trials explored IFN-γ as an adjuvant to surgery or to chemotherapy, but its success has been limited [79, 80] likely due to the i) absence of tumor immunogenicity, ii) IFN-γ-signaling components loss, iii) upregulation of IFN-γ signaling inhibitors, iv) immunosuppressive tumor microenvironment, v) failure to deliver it locally or with the adequate periodicity to achieve a therapeutic effect and, vi) in some cases, toxicity. Noticeably, in our study these problems were minimized since 4T1 cells were previously verified to respond to IFN-γ stimulation and

its administration in *in vivo* was subcutaneous near the tumor. Nevertheless, these limitations should be addressed to improve the response to IFN-γ-based therapies. To overcome these issues, patients' stratification to identify the responders to the IFN-γ-based therapy could be helpful, as well as the combination with adjuvants could improve the tumor immunogenicity. In addition, the delivery and safety problems associated with IFN-γ administration, can be solved using nanomedicine-based strategies.

To improve the delivery of IFN-γ and decrease the previous reported toxicity effects, we decide to explore Ch/γ-PGA NPs as carriers for IFN-γ, as described in the chapter VII. In fact, several restrictions inherent to IFN-γ molecular properties have been described. Basically, these include stability problems, and tendency to aggregate irreversibly under mild denaturing conditions, with subsequent loss of biological activity [81]. In addition, IFN-γ when administered intravenously is quickly cleared [82], demanding frequent administrations of high cytokine concentrations, to induce an effective response at the target site, while leading to systemic toxicity and side effects, such as fever, fatigue, nausea, vomiting, diarrhea, neurotoxicity, and leukopenia [83]. In our study, we successfully incorporated IFN-γ on Ch/γ-PGA NPs but the biological impact was less evident than the expected from our previous *in vitro* and *in vivo* studies (chapter VI), where we used soluble IFN-γ. Additionally, we had previously demonstrated that IFN-γ release from polyelectrolyte multilayer films (PEMs) of Ch and γ-PGA inhibited M2 macrophage profile [84]. Still, the incorporation of IFN-γ on Ch/γ-PGA NPs potentiated the CD86 expression by macrophages and CD40 by DCs, and further decreased the ability of APCs to induce cancer cell invasion. To further complement this study, a pilot experiment was performed to test IFN-γ-NPs ability to inhibit tumor growth in orthotopic breast tumor model. Preliminary data, not included in this thesis, demonstrated that IFN-γ-NPs did not confer an advantage in the impairment of tumor growth in comparison with Ch/γ-PGA NPs. In addition, we could not demonstrate the activation of IFN-γ receptor, indicating that this strategy requires further optimization. To improve and sustain the effects of this delivery system, we should exploit other solutions as incorporating higher amounts of IFN-γ, for instance, consider IFN-γ incorporation into nanocapsules of Ch/γ-PGA [85].

Overall, this PhD work contributed to develop an immunomodulatory strategy, able to reprogram innate immune cells profile and function *in vitro.* Additionally, this strategy was demonstrated to synergize with conventional anticancer therapy and with IFN-γ to boost antitumor immunity in a poor immunogenic breast tumor model. The potential of Ch/γ-PGA NPs as nanocarriers was also demonstrated. At the same time, this work resulted in a series of unanswered questions which require further research in the future. Possibly, the immune

cell status of the tumor microenvironment, rather than its abundancy, can dictate the tumor outcome, opening perspectives to effective strategies for immune reprogramming.

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