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MOLECULAR BASIS OF THE EFFECT OF RADIOTHERAPY ON MACROPHAGE AND ON MACROPHAGE/CANCER CELL CROSSTALK

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É preciso … mesmo sem ver, Acreditar!

Mariza em “Melhor de Mim” (Mundo©2015)

To all of those who always believed!

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Abstract

Malignant tumours are much more than solely proliferating masses of genetically altered cells with invasive capacities. Cancer cells are surrounded by several stromal cellular components, like immune cells, endothelial cells and fibroblasts, supported by an extensive network of macromolecules named extracellular matrix. Overall, this complex ecosystem, termed tumour microenvironment, contributes to almost every hallmarks of cancer, thereby promoting tumour progression. Particularly, immune cells constitute a heterogeneous population, whose different subpopulations may contribute to either tumour progression or suppression, depending on the molecular characteristics found at the surrounding microenvironment. Our team is particularly interested on macrophages and on their role in colorectal cancer progression. Importantly, macrophages are also described to promote resistance to therapy, including radiotherapy, an important anti-cancer treatment used in almost 50% of all cancer patients. Despite all the technological advances and the unquestionable role of radiotherapy in local tumour control, namely in rectal cancer, resistance to therapy is still a major challenge in cancer treatment. In order to achieve the desirable efficacy, strategies combining radiotherapy with the modulation of tumour immunity have emerged.

The present PhD work aims to contribute with new insights into the comprehension of ionizing radiation effects on: i) macrophage and on ii) macrophage-cancer cell crosstalk. To address these issues, macrophages and cancer cells were irradiated with clinically relevant doses (2 Gy/fraction/day) during 5 days, mimicking a week of cancer patient´s radiation treatment. Characterization of macrophage response to ionizing radiation, at a functional level, demonstrated that despite DNA damage, irradiated macrophages are viable and remain metabolically active, possibly through activation of NF-κB RelB subunit and through increase of Bcl-xL expression. Notably, ionizing radiation seems to induce a mixed macrophage functional phenotype: promoting the increase of macrophage pro-inflammatory markers, like *CD80*, CD86 and HLA-DR, and reducing the expression of anti-inflammatory ones, like IL-10, *VCAN* and CD163. Although without inducing an increased expression of typical pro-inflammatory markers, such as *TNF*, *IL1B* or IL-12, ionizing radiation maintains the ability of macrophages to promote cancer cell invasion and cancer cell-induced angiogenesis, similarly to non-irradiated macrophages. Additionally, a broader characterization of macrophage response to radiation, using proteomic tools, allowed us to identify the main signalling pathways and targets altered by radiation exposure. Our results demonstrated that irradiated macrophages exhibit downregulation of cathepsin D, a lysosomal protease involved in antigen processing and presentation, and

upregulation of transferrin receptor 1, which is involved in cellular iron uptake through its interaction with transferrin, an iron-binding protein. These alterations may have implications in the macrophage radiation resistant profile described in the literature, although further investigations are still required.

To study the effect of ionizing radiation on macrophage-cancer cell crosstalk, indirect Transwell co-cultures were established and subsequently irradiated. Results demonstrated that macrophages increase the radioresistance of SW1463 cancer cells, while promoting RKO radiosensitivity, suggesting the need for different biological strategies to treat radiosensitive or radioresistant tumours. Additionally, the expression profile of macrophages irradiated, alone or in co-culture, evidenced the ability of cancer cells to modulate macrophage response to radiation, inducing their differentiation into a more pro-inflammatory profile. Finally, our data suggested that attention should also be paid to the non-target effects of radiotherapy, as non-irradiated RKO cancer cells exhibit increased invasion and migration upon stimulation with conditioned medium from irradiated co-cultures.

Overall, the present work contributes with new insights into the comprehension of the response of both macrophages and cancer cells to ionizing radiation. Importantly, the acquired knowledge as well as the discovery of potential new molecular targets may be relevant for the design of further strategies complementary to radiotherapy, aiming at circumvent macrophage-mediated mechanisms of tumour radioresistance.

Resumo

Os tumores malignos são estruturas mais complexas do que apenas massas proliferativas de células geneticamente alteradas com capacidades invasivas. As células tumorais desenvolvem-se rodeadas de vários componentes celulares do estroma, como células imunes, células endoteliais e fibroblastos, sustentados por uma extensa rede de macromoléculas designada de matriz extracelular. No seu conjunto, este complexo ecossistema, designado por microambiente tumoral, modela as atividades das células tumorais, regulando assim a progressão tumoral. Em particular, as células imunes constituem uma população heterogénea, composta por diferentes subpopulações que, dependendo do contexto molecular que as rodeia, podem contribuir tanto para a progressão como para a supressão tumoral. De entre as células imunes, a nossa equipa está particularmente interessada nos macrófagos e no seu papel na progressão do cancro colorectal. Os macrófagos também promovem a resistência à terapia, em particular à radioterapia, um importante tratamento usado em cerca de 50% de todos os doentes com cancro. Apesar dos avanços tecnológicos e do inquestionável papel da radioterapia no controlo local do tumor, nomeadamente no cancro rectal, a resistência à terapia continua a ser o maior desafio do tratamento oncológico. Para obter a eficácia desejada, têm emergido estratégias que combinam a radioterapia com a modulação da imunidade tumoral.

O presente trabalho de doutoramento pretende contribuir com uma visão mais aprofundada para a compreensão dos efeitos da radioterapia: i) nos macrófagos e ii) na comunicação entre macrófagos e células tumorais. Para responder a estas questões, ambas as populações foram irradiadas com doses clinicamente relevantes (2 Gy/fração/dia), durante 5 dias, mimetizando uma semana do tratamento com radiação ionizante de um doente com cancro. A caracterização da resposta dos macrófagos à radiação demonstrou que, apesar do dano no DNA, os macrófagos irradiados são viáveis e mantêm a sua atividade metabólica, exibindo um aumento da sobrevida, possivelmente através da ativação da subunidade RelB do NF-κB e do aumento de expressão da Bcl-xL. A radiação ionizante parece induzir um fenótipo funcional misto nos macrófagos, promovendo um aumento de marcadores pro-inflamatórios como *CD80*, CD86 e HLA-DR, e reduzindo a expressão de marcadores anti-inflamatórios, como IL-10, *VCAN* e CD163. Embora sem induzir um aumento de expressão de marcadores pro-inflamatórios típicos como *TNF*, *IL1B* ou IL-12, a radiação ionizante mantém a capacidade dos macrófagos promoverem a invasão das células cancerígenas bem como da angiogénese por elas induzida, de modo semelhante aos macrófagos não irradiados. Além disso, uma caracterização mais abrangente da resposta dos macrófagos à radiação, recorrendo a ferramentas de proteómica, permitiu-nos identificar as principais vias de

sinalização e alvos alterados pela exposição à radiação. Os resultados demonstraram que os macrófagos irradiados exibem uma diminuição da expressão da catepsina D, uma protease lisossomal envolvida no processamento e apresentação de antigénios, e um aumento da expressão do receptor 1 da transferrina, que está envolvido na absorção de ferro através da interação com a transferrina, que é uma proteína que se liga ao ferro. Estas alterações podem ter implicações no perfil de radioresistência dos macrófagos, apesar de serem necessários estudos adicionais.

O estudo do efeito da radiação na comunicação macrófago-célula tumoral envolveu o estabelecimento e posterior irradiação de co-culturas indiretas em Transwells. Os resultados demonstraram que os macrófagos aumentam a radioresistência das células cancerígenas SW1463, enquanto promovem a radiosensibilidade das RKO, sugerindo a necessidade de estratégias diferentes para o tratamento de tumores radiosensíveis ou radioresistentes. Além disso, o perfil de expressão de marcadores pró- e anti-inflamatórios nos macrófagos irradiados, sozinhos ou em co-cultura, evidenciou a capacidade das células cancerígenas modularem a resposta dos macrófagos à radiação. Finalmente, os nossos dados salientam também os efeitos da radioterapia fora do alvo da radiação, uma vez que células cancerígenas RKO não irradiadas exibem invasão e migração aumentadas após estimulação com meio condicionado das co-culturas irradiadas.

Em suma, o presente trabalho contribui para a compreensão da resposta dos macrófagos e das células cancerígenas à radiação ionizante. De modo igualmente importante, o conhecimento adquirido, bem como a descoberta de potenciais alvos moleculares podem ser relevantes no desenho de futuras estratégias terapêuticas complementares à radioterapia, que tencionem contornar os mecanismos de radioresistência tumoral mediados pelos macrófagos.

Preface

The present PhD thesis comprises six chapters, preceded by a summary, both in Portuguese and English, and an abbreviation list.

The first chapter includes a general introduction, based on literature review, which facilitates the comprehension of the research findings described in the following chapters. Five main topics are depicted along this introduction (**Chapter I**). In the first topic, an overview of cancer as a major health concern and a genetic-based disease is addressed, with particular emphasis on the hallmarks of cancer, and on colorectal cancer as a model of study. The characterization of the tumour microenvironment as a complex network of non-cancer cells and extracellular matrix components, surrounding and supporting cancer cell activities is presented, as well as its contribution to the hallmarks of cancer. Particularly, the dual role of immune cells in cancer progression is here reviewed and clarified. In the second topic, a comprehensive description about macrophage origin, main functions, polarization status, and the role of tumour-associated macrophages in cancer progression are discussed. The following topics are dedicated to the fields of radiotherapy and radiobiology. In the third topic, a brief overview of radiotherapy in the management of cancer is presented, together with the explanation of some introductory concepts related with the type of ionizing radiation used, the therapeutic ratio definition and the use of fractionated instead of single dose regimens. Additional emphasis is given to the role of radiotherapy in rectal cancer management. The fourth topic is focused on the response of cancer and non-cancer cells, namely macrophages, to radiation exposure, highlighting the principle signalling pathways activated at the irradiated tumour microenvironment. This chapter finishes with a brief overview of some biological strategies, focused on tumour microenvironment targets, aiming to radiosensitize tumours, thereby improving radiotherapy efficacy. Although macrophage targeting or modulation, as a form of immunotherapy, was not addressed by our experimental work, we believe that the combination of this immunomodulatory strategy with radiotherapy has great potential and would certainly be the next step of this project.

Following the introduction, the aims of this PhD thesis are presented in **Chapter II**. The content of **Chapters III to V** comprises three first-author research articles, numbered in the text as 1-3, together with some complementary and unpublished data, which is based on the experimental work performed along this PhD project. Research work will be followed by the concluding remarks and future perspectives presented in **Chapter VI**, and finally by the annexes.

List of publications

A list of publications resulting from this PhD project is provided below:

Research article 1 – presented in Chapter III

Pinto, AT; Pinto, ML; Cardoso, AP; Monteiro, C; Pinto, MT; Maia, AF; Castro, P; Figueira, R; Monteiro, A; Marques, M; Mareel, M; Dos Santos, SG; Seruca, R; Barbosa, MA; Rocha, S; Oliveira, MJ. Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. *Scientific reports* **2016,** *6*, 18765.

Research article 2 – presented in Chapter IV

Pinto, AT; Osório, H; Vitorino, R; Pinto, ML; Sousa, JLR; Cardoso, AP; Lima, J; Santa, C; Manadas, B; Figueira, R; Monteiro, A; Marques, M; Seruca, R; Barbosa, MA; Mareel, M; Rocha, S; Oliveira, MJ Proteomic signature of human macrophages exposed to clinical ionizing radiation doses. Revised version resubmitted to *Journal of Proteome Research* **2016.**

Research article 3 – presented in Chapter V

Pinto, AT; Pinto, ML; Velho, S; Pinto, MT; Cardoso, AP; Figueira, R; Monteiro, A; Marques, M; Seruca, R; Barbosa, MA; Mareel, M; Oliveira, MJ*; Rocha, S*. Intricate macrophage-colorectal cancer cell communication in response to radiation. Manuscript conditionally accepted at *PLOS ONE* **2016**.

**Equally contributed senior co-authors.*

Review article

The current state-of-the-art regarding the response of macrophages to ionizing radiation exposure and the signalling pathways associated with cancer cell radioresistance, as presented in the introduction (Chapter I) of this PhD thesis, will be used for a Review article.

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Abbreviation list

2-DE - two-dimensional gel electrophoresis
5-FU - 5-fluorouracil
Akt - v-Akt murine thymoma viral oncogene homolog
ANG-2 - angiopoietin-2
APC - adenomatous polyposis coli
APCs - antigen presenting cells
ASMase - acid sphingomyelinase
ATM - ataxia telangiectasia mutated
ATR - ATM Rad3-related
BAX - BCL2-Associated X Protein
Bcl-2 - B-cell lymphoma 2
Bcl-xL - B-cell lymphoma extra long
bFGF - basic fibroblast growth factor
Bim - Bcl2-interacting mediator of cell death
BMDCs - bone marrow-derived cells
BRAF - v-raf murine sarcoma viral oncogene homolog B1
CAM - chorioallantoid membrane
CATD - cathepsin D
CD - cluster of differentiation
Cdc25A - cell division cycle 25A
Chk - checkpoint kinase
CIN - chromosomal instability
CM - conditioned medium
CRC - colorectal cancer
CSF-1 - colony-stimulating factor 1 (also known as M-CSF)
CSF-1R - CSF-1 receptor (also known as M-CSFR or CD115)
CT - chemotherapy
CTLA-4 - cytotoxic T-lymphocyte antigen 4
DAMPs - damage-associated molecular patterns
DC - dendritic cell
DNA-PKcs - DNA-dependent protein kinase catalytic subunit
DSB - double-strand break
ECM - extracellular matrix
EGFR - epidermal growth factor receptor
ELISA - enzyme-linked immunosorbent assay
ERK - extracellular signal-regulated kinase
FAK - focal adhesion kinase
FGF - fibroblast growth factor
Glut-1 - glucose transporter-1
GM-CSF - granulocyte-macrophage-CSF
GO - gene ontology
Gy - Gray (unit of ionizing radiation)
HGF - hepatocyte growth factor
HIF1α - hypoxia-inducible factor 1α
HLA-DR - human leukocyte antigen – antigen D related
ICAM-1 - intercellular adhesion molecule-1 (also known as CD54)
ICD - immunogenic cell death
IFN-γ - interferon-gamma
IGF - insulin-like growth factor
IGF2R - insulin-like growth factor 2 receptor
IGRT - image-guided radiotherapy
IL - interleukin
IMRT - intensity-modulated radiotherapy
iNOS - inducible NOS
iTRAQ - isobaric tag for relative and absolute quantitation

IκB - inhibitor of NF-κB

KRAS - Kirsten rat sarcoma 2 viral oncogene

LDHA - lactate dehydrogenase A

LD-RT - low-dose radiotherapy

LPS - lipopolysaccharide

MALDI-TOF - matrix assisted laser desorption/ionization time-of-flight

MAPK - mitogen-activated protein kinase

Mcl-1 - myeloid cell leukaemia 1

M-CSF - macrophage colony stimulating factor

MDSCs - myeloid-derived suppressor cells

MHC - major histocompatibility complex

MMP - matrix metalloproteinase

MnSOD - manganese superoxide dismutase

MR - mannose receptor

MSCs - mesenchymal stem cells

MSI - microsatellite instability

MVD - microvascular density

NF-κB - nuclear factor NF-kappa-B -light-chain-enhancer of activated B cells

NK - natural killer

NO - nitric oxide

NOS - nitric oxide synthase

PARP - poly (ADP-Ribose) polymerase

PD-1 - programmed cell death protein-1

PDGF – platelet-derived growth factor

PI3K - phosphoinositide 3-kinase

PIGF - placental growth factor

PMA - phorbol myristate acetate

PRR - pattern-recognition receptor

RAS - rat sarcoma viral oncogene

RNS - reactive nitrogen species

ROI - reactive oxygen intermediates

ROS - reactive oxygen species

RT - radiotherapy

RTKs - receptor tyrosine kinases

SDF-1 - stromal cell-derived factor-1 (also known as CXCL12)

SMAD4 - Sma- and MAD-related protein 4

SOD - superoxide dismutase

STAT - signal transducer and activator of transcription

STRING - search tool for the retrieval of Interacting Genes/Proteins

TAMs - tumour-associated macrophages

TGFBR2 - TGF-β receptor 2

TGF-β - transforming growth factor beta

Th cells - T helper cells

TLR - Toll-like receptor

TNF-α - tumour necrosis factor alpha

TP53 - tumour protein P53

TRAIL- TNF-related apoptosis-inducing ligand

Treg - regulatory T cells

VATD - V-type proton ATPase subunit D

VCAM-1 - vascular cell adhesion molecule 1

VEGF - vascular endothelial growth factor

XIAP - X-linked inhibitor of apoptosis

Chapter I

Introduction

1 Cancer

Among other chronic diseases, cancer is still one of the most common concerning health issues worldwide, particularly in more developed regions[1, 2]. Despite the high number of new cases per year, patients´ five-year survival and disease-associated mortality rate have improved over the last decades. These achievements were only possible due to important advances in several fields, namely cancer prevention, screening, patient care and treatment[3].

1.1 Colorectal cancer

Colorectal cancer (CRC) constitutes an example of extensive reduction of overall death rate, mainly due to screening programs, which improved cancer diagnosis at earlier and more treatable stages[4]. Advances in surgery and chemotherapeutic agents have also been crucial to this success. Despite such improvements, colorectal cancer is still the 3th most common cancer in men and the 2nd in women worldwide, being the 3rd most frequent type of cancer and the second cause of cancer-related deaths in Portugal[1]. To achieve reduced incidence and mortality rates, we need to improve our understanding on why some patients respond well to therapy while others do not, and why some relapse[5]. Molecular biology could be an important tool to address these questions as it allows the discovery of new biomarkers for early detection and risk stratification (diagnostic markers), prognosis (prognostic markers) and the prediction of treatment responses (predictive markers)[6].

To understand the particular biology of colorectal cancer, it is important to consider that cancers are driven by mutations, which confer them a selective growth advantage[7]. Driver mutations usually occur in pathways that regulate cell survival (like RAS, PI3K, STAT, MAPK, TGF-β, cell cycle/apoptosis signalling), cell fate (like Notch and APC), and genomic maintenance (such as ATM and p53)[7]. Although genetic alterations have a preponderant role in initiation and development of all cancers, they have been particularly well studied in CRC, which has indeed been considered an optimal model to study malignant progression from a genetic perspective[8]. Although most of the CRC cases are of the sporadic type, the identified gene mutations, which account only for 5 to 6% of the inheritable cases[8], have allowed the characterization of a well-defined sequence of genetic events, from normal colon epithelium to colorectal carcinoma (Figure 1).

The genomic instability in colorectal cancer is predominantly caused by two well-known factors: a) microsatellite instability (MSI), which is mainly caused by inactivation of genes from the mismatch repair (MMR) signalling, a key pathway in maintenance of genomic stability[9], and b)

chromosomal instability (CIN), which is characterized by the dramatic loss/gain of chromosomal material. Of all CRCs, 80 to 85 % are microsatellite stable (MSS), but most are characterized by CIN, while the remaining 15 to 20 % display MSI[10].

Classically, mutations or loss of *APC* are described to mediate the transition from normal epithelium to early adenoma and aberrant crypt formation, while the transition towards intermediate adenoma might be associated with *KRAS* mutations (in about 50% of the cases) (Figure 1). The loss of chromosome 18q, the mutated *CDC4*, a regulator of the ubiquitin-proteasome pathway, and of the mutated *SMAD4*, a downstream target of transforming growth factor beta (TGF-β), may then conduce to late adenomas. Mutations or loss of *TP53* are generally a latter event, frequently associated with the transition from adenoma to carcinoma[10, 11]. In CRCs exhibiting MSI, alterations in the Wnt signalling are generally the initial step to develop early adenomas, followed by mutations in other genes that contribute to cancer progression (Figure 1).

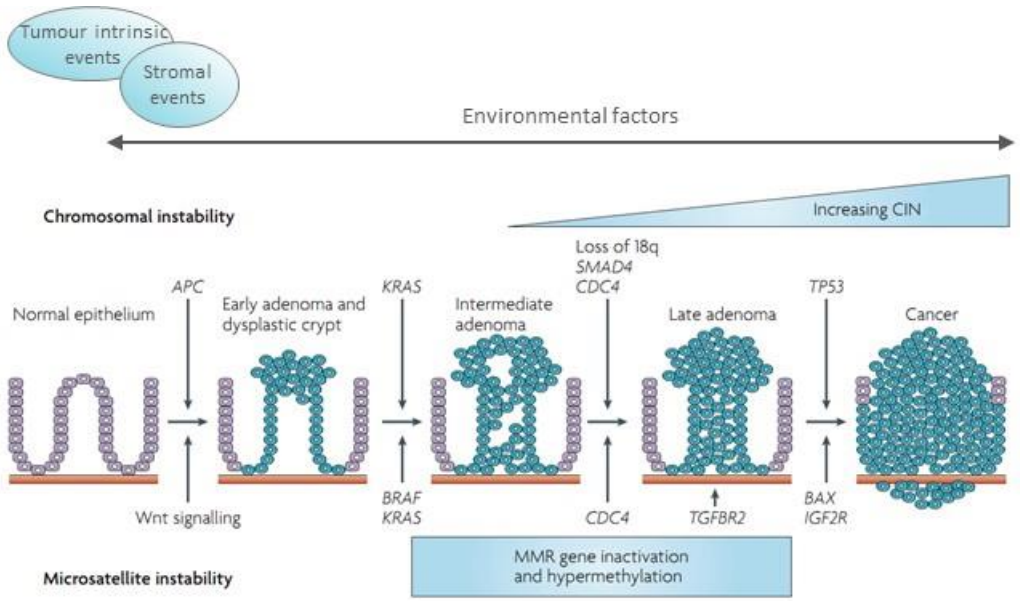

Figure 1 - Progression model of a normal colon epithelium into a cancer through genetic alterations. Accumulation of abnormalities in particular genes transforms normal epithelium through adenoma to colorectal carcinoma. Non-genetic factors, as environmental factors, tumour intrinsic or stromal events also contribute to colorectal cancer progression[8]. Abbreviations: Akt - PI3K/V-Akt murine thymoma viral oncogene homolog; APC - adenomatous polyposis coli; BRAF - v-raf murine sarcoma viral oncogene homolog B1; CDC4 - cell division control protein 4; IGF2R - insulin-like growth factor 2 receptor; KRAS - Kirsten rat sarcoma viral oncogene homolog; MLH1 - MutL Homolog 1; MMR - mismatch repair; BAX - BCL2-Associated X Protein; PTEN - Phosphatase and Tensin homolog; SMAD4 - Sma- and MAD-related protein 4. Adapted by permission from Macmillan Publishers Ltd: [Nature][5], copyright (2009).

As genomic studies confirm, CRC cell lines harbour similar number and type of mutations as those commonly described in CRC primary tumours, being therefore considered valuable *in vitro* tools

to investigate CRC cellular and molecular events, as well as response to treatment[12, 13]. Genetic alterations of some of the most common human CRC cell lines are presented in the Annexes section of the present document.

Due to their importance in CRC progression, some of the genetic alterations above mentioned have potential as prognostic and predictive biomarkers in CRC[5, 14]. Currently, only mutated *KRAS*, *BRAF*, MSI and the genomic test Oncotype *DX*® are used as biomarkers in clinical practice. The first two lead to constitutive activation of the epidermal growth factor receptor (EGFR) signalling pathway, being predictive markers of resistance to anti-EGFR monoclonal antibody therapy in metastatic CRC patients, although *KRAS* wild-type cancers acquire resistance after initial response[15, 16]. MSI is a prognostic biomarker identifying patients with the most common CRC predisposing syndromes – the Heritable Non-Polyposis Colon Cancer (HNPCC)[15]. However, finding effective predictors of response to radiotherapy, another anti-cancer therapy, is challenging and no biomarkers are currently available in the clinical practise[14]. Although some studies demonstrate an association of mutated *TP53* with radioresistance and MSI or lack/mutated ATM may predict increased radiosensitivity, response to radiotherapy is highly variable among cancer patients[17, 18]. Contradictory reports and lack of translational studies difficult the discovery of predictive markers for radiotherapy response in CRC.

1.2 Hallmarks of cancer

Although genetic instability is recognized as crucial to achieve a malignant phenotype, cancer cells also acquire other capabilities, overall designated as hallmarks of cancer[19], which confer them growth advantage, increased survival and proliferation as well as dissemination ability. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer: *sustaining proliferative signalling*, *evading growth suppressors*, *resisting cell death*, *enabling replicative immortality*, *inducing angiogenesis*, and *activating invasion and metastasis*. Later on, an update to these six original hallmarks of cancer considered *genome instability & mutation*, together with *tumour promoting-inflammation*, as transversal properties to most of the core hallmarks, being therefore termed as enabling characteristics of cancer[20]. At the same time, *reprogramming of energy metabolism* and *evading immune destruction* were also recognized as two emerging hallmarks of cancer. The characterization of each hallmark, as well as some strategies acquired by cancer cells to develop these abilities, will be here explained.

Sustaining Proliferative Signalling

Contrarily to normal cells, cancer cells are able to sustain proliferative signalling, which can be achieved through: i) the production of growth factors, which stimulate cancer cell proliferation in an autocrine or paracrine manner; ii) structural alterations or increased expression of growth receptors, which may render cancer cells hyperresponsive to growth factor stimulation; and iii) alterations in downstream targets of these growth factor receptors, which may lead to constitutively active proliferative signalling pathways and consequently to chronic proliferation[20]. The ability to sustain proliferation is frequently acquired through mutations, for instance in *BRAF* and *PI3K*, which result in constitute activation of MAPK and PI3K proliferation-associated signalling pathways, respectively[21, 22].

Evading Growth Suppressors

To be able to successfully sustain proliferative signalling, cancer cells have also to overcome the action of tumour suppressors, such as the retinoblastoma (RB) protein and the p53 transcription factor, which limit cell growth and proliferation by preventing cell-cycle progression or by activating senescence and apoptotic programs in case of cell stress and genomic damage[20, 23]. Inactivating mutations in *TP53* and defects in Rb pathway endow cancer cells with growth and survival advantages[20, 24]. Also TGF-β signalling plays a central role in proliferation inhibition, and alterations in *TGFBR2* are particularly common in CRC[25].

Resisting Cell Death

In order to maintain survival, even upon exposure to damaging stimuli, cancer cells developed several strategies to avoid apoptosis, like: i) loss of damage sensor *TP53*; ii) increased expression of anti-apoptotic regulators (such as B cell lymphoma 2 (Bcl-2) and B cell lymphoma extra-long (Bcl-xL)) or of survival signals (insulin-like growth factor 1 and 2 (IGF1, IGF2), iii) downregulation of pro-apoptotic factors (like Bax and Bcl2-Interacting Mediator of cell death (Bim)), or iv) by short-circuiting the extrinsic ligand-induced death pathway[20]. To continue supporting survival, cancer cells also need to circumvent autophagy, although depending on the stress level, its promotion may indeed be protective through removal of damaged proteins and organelles[26, 27]. Additionally, necrosis activation is clearly beneficial for cancer cells, as it induces the release of pro-inflammatory signals, which then recruit inflammatory immune cells that provide growth-stimulating factors[20, 28].

Enabling Replicative Immortality

Contrarily to normal cells, in which telomere shortening limits the number of viable cellular divisions, cancer cells exhibit unlimited replicative potential, mainly due to increased expression of telomerase, which is the enzyme complex responsible for elongating telomeres after each cell division[20, 29].

Inducing Angiogenesis

Although in adult normal cells angiogenesis is turned off most of the time, in cancer cells there is an "angiogenic switch" that allows a permanent induction of angiogenesis, required to support increased nutrient and oxygen needs, as well as higher evacuation of metabolic wastes and carbon dioxide. Chronic angiogenesis, mainly induced by upregulation of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)[20, 30], leads to an abnormal neovasculature, characterized by irregular shape with distorted and enlarged vessels, excessive vessel branching, erratic blood flow, microhaemorrhaging and lack of the normal hierarchical arrangement of arterioles, capillaries and venules[31].

Activating Invasion and Metastasis

Cancer invasion and metastasis are important properties of cancer cells, transforming a locally growing tumour into a systemic, metastatic, and live-threatening disease[32]. Although not unique to cancer cells, invasion describes the ability to penetrate tissue barriers, such as basement membrane and interstitial stroma, which is intimately related with adhesion, proteolysis of extracellular matrix (ECM) components and cell migration[33]. Loss of E-cadherin, a key molecule in cell-to-cell adhesion, is one of the best characterized alterations of invasive cancer cells[20]. Local invasion and migration of cancer cells into nearby tissues constitute the initial steps of metastization, which also involves cancer cell intravasation into nearby blood and lymphatic vessels, survival in circulation and escape into the parenchyma of distant tissues (extravasation), where they finally seed (micrometastases) and "colonize" a new niche[34].

Enabling characteristics:

Genomic instability & mutation

As exemplified in the Chapter 1.1, dedicated to mutations in CRC, genetic alterations in cancer cells are fundamental for cancer progression and response to therapy.

Tumour-promoting inflammation

This property reflects the increased influx of inflammatory cells observed in tumours, which was initially seen as an attempt to eradicate cancer cells, but paradoxically revealed to be associated with cancer progression[20]. The dual role of immune cells in cancer will be further discussed.

Emerging hallmarks:

Reprograming cancer cellular metabolism

Cancer cells adopt a different metabolic strategy from normal and differentiated cells, by relying mainly on aerobic glycolysis instead of oxidative phosphorylation, a phenomenon known as "Warburg effect" [35, 36]. Despite the resulting lower ATP efficiency, cancer cells may benefit from this strategy, as high glycolysis rate may favour the synthesis of intermediate molecules, important for the formation of new organelles and macromolecules, and thus essential for new cell assembly[20]. Upregulation of the glucose transporter 1 (GLUT-1) and lactate dehydrogenase A (LDHA) are important for this switch, allowing a higher glucose import into the cytoplasm as well as a more rapid conversion of pyruvate, resulting from glycolysis, into lactate[37, 38].

Escaping from immune system

This ability consists in escaping from immune system attack, particularly from killing T cells[20].

1.3 The tumour microenvironment

In 1970s, the notion that it was required an appropriate environment for the development of a malignant phenotype was first explored[39]. Since then, particular attention has been paid to the characterization of the tumour microenvironment where tumour cells arise and develop. It is now well-accepted that, particularly solid tumours, constitute highly complexes ecosystems involving the communication between three main components: tumour cells, tumour-associated host cells and elements of the extracellular matrix (ECM), the complex network of macromolecules that supports tissue architecture and integrity during homeostasis (Figure 2)[40].

Tumour-associated host cells comprise blood and lymphatic endothelial cells, pericytes, fibroblasts and a variety of bone marrow-derived cells (BMDCs): macrophages, TIE2-expressing monocytes (TEMs), lymphocytes, neutrophils, mast cells, myeloid-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs). On its turn, the ECM composition (collagen type I, collagen type IV, fibronectin, laminin), its unique physical (like rigidity, porosity, insolubility and topography), biochemical (signalling capability) and biomechanical (elasticity) properties are frequently deregulated in tumours[41].

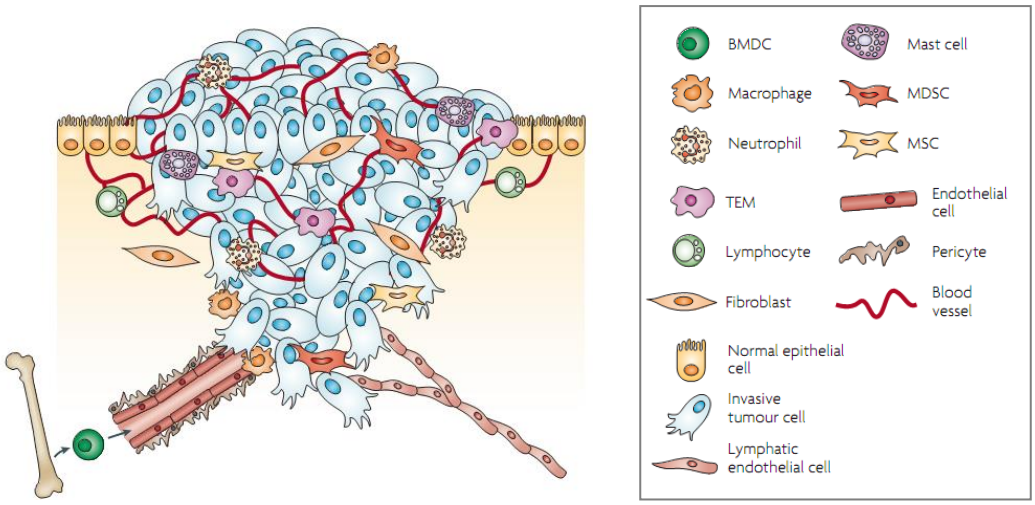

Figure 2 - The tumour microenvironment. The tumour microenvironment is an extremely rich and complex ecosystem composed by many different cells and a deregulated ECM. Altogether these elements modulate and are modulated by cancer cell activities, dictating the success of cancer progression. Adapted by permission from Macmillan Publishers Ltd: [Nature][40], copyright (2009).

1.3.1 The tumour microenvironment modulates tumour progression

The recognition of tumours as more than just a mass of genetically altered cells has improved our knowledge about tumour biology[42]. Although in normal tissues, stromal cells (essentially fibroblasts, adipocytes and immune cells) and ECM contribute to homeostasis maintenance, their deregulation may favour tumour progression[20]. Some examples of the contribution of distinct tumour microenvironment elements to several hallmarks of cancer will be briefly provided[43]. For simplicity, four main groups will be considered: angiogenic vascular cells, involving endothelial cells and their supporting pericytes; cancer-associated fibroblastic cells, comprising connective tissue fibroblasts, MSCs, myofibroblasts and activated adipocytes; infiltrating immune cells; and finally the ECM[20].

Tumour-stromal cells provide a variety of growth factors that sustain cancer cell proliferation, like EGF, TGF-β, tumour necrosis factor- α (TNF-α), FGFs, various cytokines and chemokines, hepatocyte growth factor (HGF), IGF-1 and stromal cell-derived factor-1 (SDF-1/CXCL12). Also, cancer cell adhesion to ECM promotes self-sufficient growth and survival, through extracellular signal-regulated kinase (ERK) and PI3K pathway activation[44], and collagen deposition and ECM stiffness upregulate integrin signalling, promoting cancer cell survival and proliferation[41, 45, 46]. Tumour microenvironment components help cancer cells to overcome the growth suppression observed in normal cells. For instance, contrarily to "normal" fibroblasts, cancer-associated fibroblastic cells are not able to inhibit cancer cell growth through direct cell-cell contact[43].

Infiltrating immune cells release proteolytic enzymes, like metallo, serine, and cysteine proteinases that cleave cell-cell and cell-matrix adhesion complexes[43]. Additionally, cancer cell death resistance is facilitated by the formation of new vessels by angiogenic vascular cells, increasing oxygen and nutrients supply. Also tumour-associated macrophages (TAMs) secrete molecules, like α4-integrin that binds to the vascular cell adhesion molecule 1 (VCAM-1) in cancer cells, suppressing apoptosis through induction of PI3K/AKT signalling.

Tumour-associated cells also secrete a wide range of soluble mediators involved in angiogenesis promotion, like growth factors (VEGF, basic fibroblast growth factor (bFGF), TNF-α , TGF-β , platelet-derived growth factor (PDGF), placental growth factor (PIGF)), chemokines (CXCL12, IL-8/CXCL8), matrix metalloproteinases (MMPs), histamine, and other bioactive mediators (like nitric oxide (NO)) [45]. Importantly, the balance between production and degradation of ECM, where many factors are sequestered, may potentiate the release into the interstitial fluid of multiple pro- and anti-angiogenic factors[43, 44]. Besides being involved in angiogenesis promotion, tumour-associated cells are also involved in every steps of the metastatic cascade[47], particularly mast cells and macrophages through breakdown of ECM molecules and stimulation of EGFR in tumour cells. Through VEGF and inducible nitric oxide synthase (iNOS) production, which leads to alterations in vascular tension and function and loss of pericyte coverage, endothelial cells facilitate cancer cell extravasation[45].

Finally, the immune system evasion is facilitated by i) the infiltration of immunosuppressive cells, like regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs), anti-inflammatory macrophages, neutrophils and mast cells[43]; ii) the non-permissiveness of endothelial cells to intensive T cell inflammation; and iii) the TGF-β secretion mediated by cancer-associated fibroblastic cells that inhibits cytotoxic T cells and natural killer (NK)/T cells recruitment[43].

1.3.2 Infiltrating immune cells: cancer drivers or fighters?

Infiltrating immune cells have a preponderant role in early and more advanced tumour stages, contributing to several hallmarks of cancer, as demonstrated in the previous section. The association between chronic inflammation and cancer development was first postulated by Rudolf Virchow during the 19th century, after observation of leukocyte accumulation in cancer tissues[48, 49]. Accordingly, inflammation induced by infectious organisms are nowadays associated with about 20 % of the tumours[50]. The classical example is the association of tobacco smoke with lung cancer[51], but other unquestionable cases are the association of Hepatitis B and C virus,

Helicobacter pylori and papilloma virus with the development of hepatocellular, gastric and cervical cancer, respectively[52, 53, 54]. Also inflammatory diseases are closely linked with CRC incidence[55]. For instance, patients with Crohn disease, ulcerative colitis in colon or rectum have respectively a 2.6, 2.8 and 1.9 increased risk of CRC development[56].

Although their association with cancer initiation and development, infiltrating immune cells are also able to supress cancer progression. This apparent paradox may be explained by the evolution of the type of immune populations present along different steps of tumour progression[57]. Tumour-infiltrating immune cells refer to a very broad group of cells including leukocytes from both myeloid (like macrophages, dendritic cells (DCs) and neutrophils) and lymphoid (B, T and NK cells) origins that exhibit antagonistic roles, contributing to promotion or suppression of immune response[58]. NK as well as $CD8^+$ and helper type 1 (Th1) T lymphocytes have been viewed as the traditional players in the immune response against cancer cells, due to their ability to recognize tumour antigens or to modulate their presentation by other cells, and also to secrete interferon gamma (IFN-γ)[58]. More recently, other populations with anti-tumour properties, like Th17 cells, natural killer T (NKT) and mouse γδ T cells have been defined and their heterogeneity is still under characterization. Also tumour-associated macrophages (TAMs) and tumour-associated neutrophils (TANs) are immune populations with both pro- and anti-tumour properties, depending on the stimuli and environmental context. Ultimately, MDSCs, which represent a heterogeneous population of myeloid progenitors and precursors of macrophages, granulocytes and DCs are, together with Tregs, the main groups of leukocytes harbouring immunosuppressive properties[58].

Immune recognition of tumours

Although $CD8^+$ T cells and Th1-oriented $CD4^+$ lymphocytes are commonly seen as the main players in anti-tumour immune response, this is in fact a very well-coordinated action orchestrated by several immune cells, as will be briefly explained (Figure 3)[59]. Upon stimulation, NK cells release IFN-γ, which inhibits proliferating cancer cells and angiogenesis, but also stimulates DCs to present antigens to cytotoxic $CD8^+$ T lymphocytes, and directly induces cancer cell apoptosis. Both NK and cytotoxic $CD8^+$ T cells are able to lyse cancer cells, through perforin/granzyme pathway or apoptosis-inducing ligands, such as TNF-related apoptosis-inducing ligand (TRAIL). Macrophages are also able to induce cancer cells lysis through the production of nitric oxide (NO) and reactive oxygen species (ROS). In addition, $CD4^+$ T lymphocytes can differentiate into Th1 cells, inhibiting tumour angiogenesis and inducing cancer cell apoptosis, or into Th2 cells, secreting several interleukins that enhance eosinophil function and B cell antibody production against cancer cell-

surface molecules. These antibodies stimulate tumour destruction through binding to the Fc receptors present at macrophage surface, granulocytes and NK cells.

Besides direct ways of killing cancer cells, immune cells, particularly lymphocytes and granulocytes can also attach to tumour blood vessels and destroy them, limiting oxygen and nutrient supply to tumour cells (Reviewed by[59]). Due to their dual role in tumour initiation/progression and suppression, infiltrating immune cells have been appointed as important targets for anti-cancer therapies, generally termed as cancer immunotherapy.

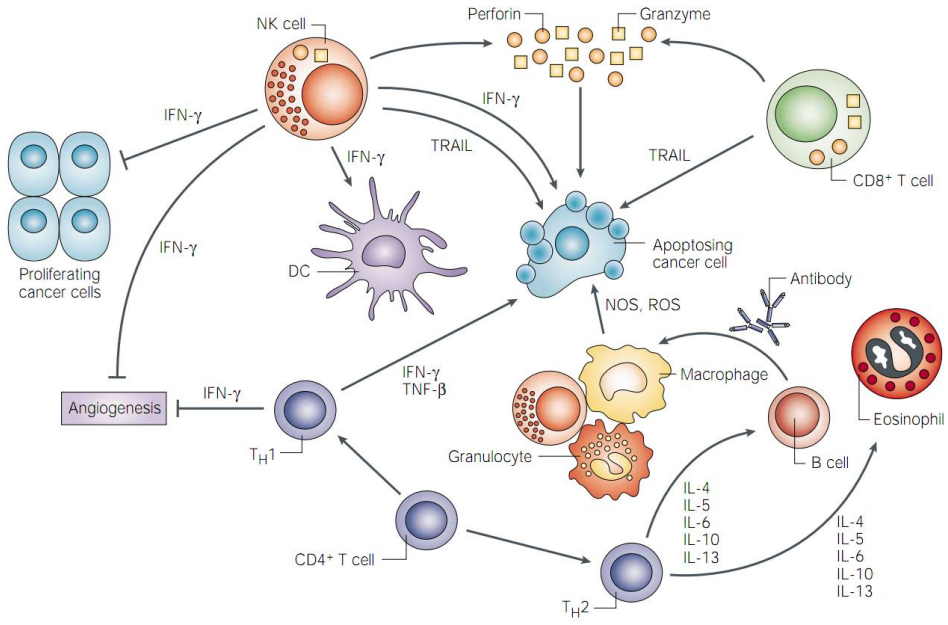

Figure 3 – The coordinated action of immune cells in tumour destruction[59]. Abbreviations: DC - dendritic cell; IFN-γ - interferon-gamma; IL - interleukin; NK - natural killer; NOS - nitric oxide synthase; ROS - reactive oxygen species; Th - T helper; TNF-β - tumour necrosis factor beta; TRAIL - TNF-related apoptosis-inducing ligand. Reprinted by permission from Macmillan Publishers Ltd: [Nature][59], copyright (2004).

2 Macrophages

Among the variety of inflammatory cells, macrophages are the most abundant leukocytes in chronic inflammatory diseases, such as asthma, as well as in many solid tumours[60, 61]. These cells literally termed as the "big eaters", in Greek, can be found in every tissues of the body, performing different functions according to tissue specialization[62]. For instance, intestinal macrophages are involved in the recognition and removal of enteric pathogens as well as tolerance to food antigens and microbiota, while bone macrophages, termed osteoclasts, are involved in bone resorption[62]. Overall, macrophages contribute to tissue homeostasis through immune surveillance activities, including phagocytosis, antigen presentation and immune suppression. Together with DCs,

macrophages and their common precursors, the monocytes, compose the mononuclear phagocyte system.

2.1 Origin and working models

The classical view of the mononuclear phagocyte system origin is well documented. Accordingly, monocytes are continuously generated in the bone marrow from haematopoietic stem cells via intermediate precursors, entering then into blood circulation and differentiating into macrophages or DCs in the peripheral tissues[63]. Particularly, tissue macrophages were believed for many years to be terminally differentiated cells without proliferation ability, derived and maintained exclusively from peripheral blood monocytes[64, 65]. As examples of relatively short-lived and non-self-renewing tissue-resident macrophages are i) those exhibiting homeostatic inflammation (located in the intestine, the remodelling mammary gland and the heart) and ii) macrophages associated with pathological inflammation, like tumour-associated macrophages (TAMs)[66]. However, in a paradigm shift, it was recently found that most of the macrophages within healthy tissues have a pre-natal (yolk sac and foetal liver) origin, exhibiting longevity and self-renewal properties[67,66,70]. Despite this apparent clear view of macrophage origin, this is still a research topic under intense investigation.

Working models

There are two preferred working models regarding *in vitro* generated macrophages, either through murine bone marrow monocytes or human peripheral-blood monocytes, both requiring exogenous colony stimulating factor 1 (CSF-1; also known as macrophage-CSF (M-CSF))[67] (Figure 4A). CSF-1 is a crucial factor known to regulate macrophage survival, proliferation and chemotaxis, through binding to its receptor (CSF-1R; also known as M-CSFR)[68]. It is released into circulation, mainly by endothelial cells, or stably expressed on the cell surface of several locally CSF1-producing cells[69, 70]. In mouse models, inactivating mutations in *CSF1* or *CSF1R* lead to a huge reduction in macrophage number as well as to macrophage abnormal features[71, 72, 73, 74].

Alternatively, murine bone marrow-derived monocytes can also be stimulated with granulocyte-macrophage-CSF (GM-CSF), but it is important to be aware that in these conditions obtained cultures may contain substantial numbers of DCs, namely $CD11c^+$ ones[67]. Additionally, macrophages *ex vivo* cultures can be obtained either through injection of mice with thioglycollate, followed by peritoneal lavages, or through extraction of infiltrating macrophages directly from mice or human tissues[67] (Figure 4B). However, isolation of macrophages from human tissues without contamination by other cell populations is a challenging procedure[67]. Importantly, the

different monocyte/macrophage isolation methodologies can be applied under normal or pathological conditions.

To evaluate the purity of isolated monocytes and confirm their differentiation into macrophages, distinct markers are frequently used. Evaluation of the expression of the monocyte/macrophage lineage antigen CD14 (lipopolysaccharide (LPS) co-receptor) and of the glycoprotein CD68 is frequently used to identify human monocytes or macrophages, respectively. The monocyte/macrophage lineage antigen CD11b (integrin αM) and the glycoprotein F4/80 are used for mouse monocytes or macrophages, respectively, being particularly useful for immunohistochemistry studies[62, 75, 76]. An overview of the main differences between murine and human macrophages will be explored along the topic "Man is not a mouse" within Chapter 2.3.

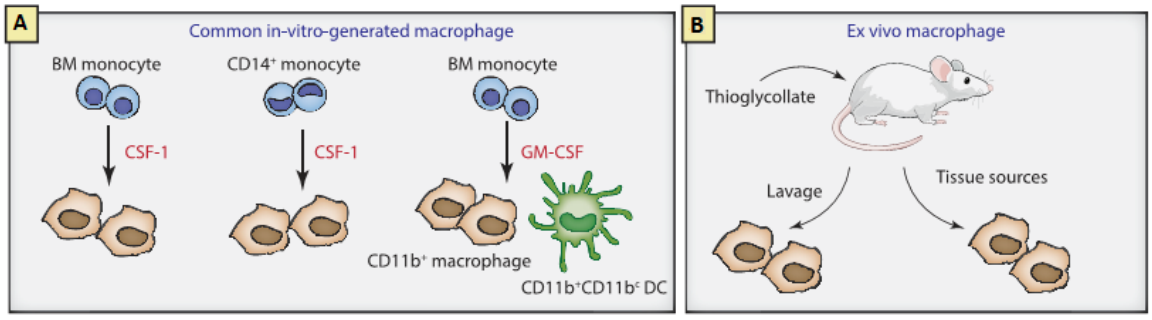

Figure 4 – Common *in vitro* and *ex vivo* macrophage working models. Adapted[67] with permission from Elsevier.

As isolation of primary monocytes and macrophage cultures can be technically demanding and expensive, requiring some level of expertise and access to adequate sources, such as human blood samples, immortalized monocyte/macrophage-like cell lines, which can be cultured easily and in unlimited amounts, have been generated using different animal species[77]. As an example, several human myeloid cell lines, such as THP-1 and U937, as well as murine macrophage-like cell lines, such as RAW 264.7 and J774A.1, are commercially available. Particularly, the THP-1 cell line, which was originally established from an acute monocytic leukaemia patient[78], is a widely used *in vitro* model for the study of monocyte-macrophage differentiation[79]. After stimulation with phorbol myristate acetate (PMA), THP-1 cells adhere to glass, a characteristic of the differentiation of monocytes into macrophages, and exhibit macrophage-like functions, such as increased phagocytosis[80]. However, attention needs to be paid when interpreting results from transformed and PMA-differentiated macrophage-like cells, as these may not be completely representative of primary human macrophages, as demonstrated by a gene expression analysis comparing macrophage from both sources[81]. Additionally, another study demonstrated that, contrarily to monocyte-derived macrophages, PMA-treated THP-1 cells are not so firmly adhered to the surface

and do not exhibit high levels of granularity and autofluorescence[82]. Despite optimization procedures to obtain a phenotype resembling monocyte-derived macrophages, PMA-treated THP-1 cells still present, for instance, high levels of constitutive TNF-α and IL-1β[82].

2.2 Macrophage functions

Macrophages exhibit homeostatic, protective or pathogenic roles in health and disease[62]. Their classical functions include phagocytosis (and efferocytosis), chemotaxis, antigen processing and presentation, and secretion of several regulatory molecules, as will be elucidated below[83].

Phagocytosis (and efferocytosis)

Phagocytosis is the engulfment of solid particles, namely invading microorganisms (like bacteria, viruses, fungi and protozoa), by pseudopodia and its digestion inside vesicles containing several molecules, like ROIs, elastase, collagenases, lipases, deoxyribonucleases, polysaccharidases, sulfatases, phosphatases and defensins[83]. Macrophages are, together with monocytes, DCs, neutrophils and mast cells, considered "professional" phagocytic cells due to their highly efficient particle internalization[62, 84]. Phagocytosis first involves the recognition of "pathogen-associated molecular patterns" (PAMPs), which are receptors on target cells (like LPS on the surface of Gram-negative bacteria), by "pattern-recognition receptors" (PRRs) present on phagocytic cells (like scavenge or mannose receptors - MRs)[84] and also rearrangements of actin cytoskeleton, which are also crucial for particle internalization. Phagocytosis of apoptotic cells is specifically termed efferocytosis. This process is important for resolution of inflammation by macrophage ingestion of apoptotic bodies, which otherwise could disintegrate and release their cytotoxic content in the intercellular space, inducing more cell death and inflammation[85].

Chemotaxis

Chemotaxis is defined as the cell movement towards a chemical stimulus, like bacterial factors, lipid mediators, ECM degradation products, cytokines and also chemokines[86, 87]. Although the majority of the cells exhibit motility, immune cells, and particularly leukocytes, are more efficient in doing so, easily moving through tissues into sites of injury[87]. Chemotaxis requires the binding of the chemoattractant to their receptors on the cell surface, which then induces migratory pathways signalling activation[88]. Although macrophages express several cytokine/chemokine receptors, like CCR1, CCR5, and CCR7[89], CSF-1 is appointed as a key regulator of macrophage migration, inducing rapid actin polymerization and increased focal complexes assembly and point contact formation after binding to CSF-1R[90].

Antigen processing and presentation

Antigen-presenting cells (APCs), such as DCs, macrophages and B cells, are involved in adaptive immune response by presenting antigens, from pathogens and tumour cells, to helper T cells ($CD4^+$ T cells)[91]. This immune response is initiated in lymph nodes, probably by DCs, but may also occur, at later stages, in non-lymphoid organs, where macrophages are mainly located[92, 93]. Exogenous antigen proteins are internalized, processed into peptides through the action of proteolytic enzymes[94], and recycled to the cell surface together with major histocompatibility complex (MHC) class II glycoproteins (human leukocyte antigens (HLA)-DR, -DP, -DQ)[83] (Figure 5).

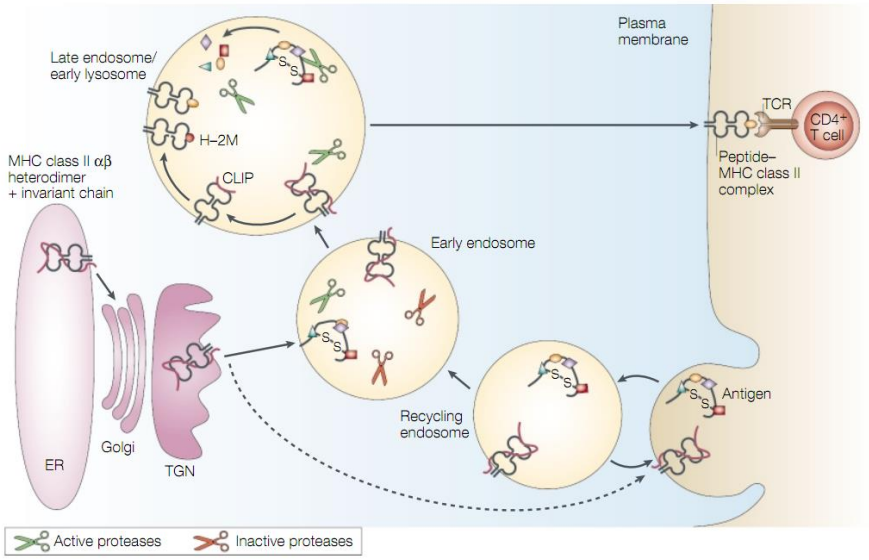

Figure 5 – The MHC-class II antigen-presenting pathway[95] . Similarly to MHC class I, MHC class II molecules are also assembled in the endoplasmic reticulum (ER) and directed with target motifs to the endosomal pathway, either directly from the trans-Golgi network (TGN) to early endosomes, or via the plasma membrane through internalization into recycling endosomes and traffic to early endosomes. Maturation of early endosomes involves its fusion with lysosomes, which are more acidic compartments, leading to pH-dependent activation of lysosomal enzymes[94]. After protein-based antigen degradation, peptides are ready to be presented to $CD4^+$ T cells in a complex with MHC class II molecules. Abbreviation: TCR – T cell receptor. Reprinted by permission from Macmillan Publishers Ltd: [Nature][95], copyright (2003).

Unlike MHC class II molecules, which expression can be induced in several cells, but is initially restricted to APCs, MHC class I molecules (HLA-A, HLA-B and HLA-C) are ubiquitously expressed and present endogenous peptides to cytotoxic T cells ($CD8^+$ T cells)[96]. Although the direct contact between APCs and T cells is required for antigen recognition, the binding of a second co-stimulatory signal(s), especially B7 proteins like CD80 (B7-1) and CD86 (B7-2), on the surface of APCs to receptors on T cells (like CD28), is crucial for $CD4^+$ T cell activation[97, 98]. Upon activation, $CD4^+$ T cells differentiate into Th1 or Th2 effector cells, eliciting pro- or anti-inflammatory responses[99].

In both phagocytosis and antigen presentation, lysosomes are crucial organelles involved in macromolecule degradation through the activity of a great variety of acid hydrolases (phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases and lipases)[100]. The best-studied lysosomal hydrolases include cathepsin proteases. Particularly, cathepsins B, D, F, K and S are expressed in macrophages and have been implicated in antigen presentation[95]. Interestingly, cytosolic translocation of activated cathepsin D is known to function as a "danger signal" alerting macrophages for the entering of invading bacteria, regulating both macrophage apoptosis and their bactericidal activity, which can be mediated through depletion of the pro-survival protein Mcl-1[101].

Secretion

Macrophages secrete a wide range of biologically active molecules[102], which are constitutively released or produced in response to appropriate stimuli, thereby regulating the activity of others cells, such as migration, and participating in several cellular processes, like immune response regulation[83]. Generally, these secretory products include enzymes (lysozyme, lysosomal acid hydrolases and neutral proteases), enzyme and cytokine inhibitors, complement components, ROS, arachidonic acid intermediates (like leukotrienes and platelet-activating factors), coagulation factors, cytokines/chemokines and others[83]. Within macrophage-released enzymes, MMPs are a family of zinc-dependent endopeptidases capable of degrading ECM components, which deserve to be highlighted due to their role in macrophage-mediated cancer cell invasion and ECM degradation (particularly MMP-2 and -9)[103]. Although not exclusively produced by macrophages, cytokines are particularly relevant as mediators of the communication between immune and non-immune cells[86] and some of the most common examples are provided in Table 1.

Table 1 – Cytokines particularly relevant for macrophage-mediated immune responses. Particularly, chemokines, a family of chemotactic cytokines, are here identified with the current nomenclature, in which the first part of the name identifies the subfamily and L stands for 'ligand' followed by a progressive number, and with the historical acronym in parenthesis[104]. Reviewed in[83, 86, 89, 105, 106].

Molecule	Description and function
IL-1-related	• A group of cytokines that comprises proteins like IL-1α, IL-1β, IL-1 receptor antagonist (Ra) and IL-18; • IL-1α and IL-1β trigger fever, induce a wide variety of acute phase response genes and activate lymphocytes; • Monocytes and macrophages are the main sources of IL-1β. Similarly to TNF, IL-1β is produced and released at early stages of the immune response to infections, lesions, and stress. Autophagy plays a major role in the release of IL-1β; • IL-18 induces IFN-γ, while decreases IL-10 production, enhances NK cell activity and promotes inflammatory Th1 cell responses. It can also attenuate IL-1β–induced fever.
IL-6	• Similar to TNF and IL-1 β, IL-6 induces fever. It also stimulates hormones, acute phase proteins and T and B cell expansion upon injury and infection; • Pleiotropic cytokine that acts as both pro-inflammatory and anti-inflammatory, affecting processes ranging from immunity to tissue repair and metabolism.
IL-10	• Plays a major role in suppressing inflammatory responses, through inhibition of TNF, IL-1β, IL-6, IL-8, IL-12, and GM-CSF produced by macrophages; also suppresses MHC-II expression in activated macrophages, being thus a potent inhibitor of antigen presentation; • Macrophage exposure to IL-10 lowers their microbicidal activity, diminishes their capacity to respond to IFN- γ, and reduces the levels of inflammatory cytokines. • Reduces the immune response against tumour cells, due to the regulatory effects on B and T cells
IL-12	• Heterodimeric cytokine comprising p35 (not active on its own) and p40 (has activity via the IL-12R) subunits; • Together with TNF and other pro-inflammatory cytokines, it stimulates IFN-γ production, as well as the cytotoxicity of NK and $CD8^+$ T cells. It plays a role in antigen presentation/processing.
IL-23	• Heterodimeric cytokine comprising p40 (common to IL-12) and p19 subunits; • IL-12 and IL-23 share the IL-12p40 subunit and both cytokines induce inflammation.
IFN	• Type I (IFN-α and IFN-β) – is secreted by virus-infected cells and leads to increased expression of MHC class I and cytotoxic T cell mobilization; • Type II (IFN-γ) – major factor converting macrophages from a "resting" to an "activated" state, which leads to increased tumour cell cytoxicity, antimicrobial activity, antigen processing and presentation through induction of MHC class II antigens.
TNF-α	• Belongs to TNF cytokine family, which is well-known by its cell death effector members; • Stimulates the acute phase of the immune response and up-regulates MHC-I and II expression.
TGF-β	• A cytokine family that includes several members, such as TGF-β1, TGF-β2 and TGF- β3, being TGF-β1 isoform the most common in immune cells; • Together with IL-10, TGF-β is another powerful anti-inflammatory cytokine downregulating the inflammatory effects of TNF, IL-1β, IL-2 and IL-12; • It is a potent suppressor of both Th1 and Th2 cells, but foments the maintenance and function of Tregs; • It plays a role in fibrosis and wound healing *in vivo*.
CXCL8 (IL-8)	• Potent chemoattractant for neutrophils; • Important role in cancer.
CCL2 (MCP-1)	• It is highly produced in response to LPS.
CCL5 (RANTES)	• Inflammatory chemoattractant for T cells, basophils, eosinophils, and DCs to the site of inflammation. • Also promotes tumorigenesis and metastasis.

2.3 Macrophage polarization

Tissue macrophages are highly plastic cells, able to reprogram their function in response to a variety of microenvironment signals[89]. Macrophage plasticity is an important property, being considered the key concept for the development of a macrophage classification system, based on the expression of certain markers and development of specific functions, upon exposure to certain stimuli[89].

In 2000, Mills and colleagues proposed the M1-M2 terminology, resembling the Th1 and Th2 responses, as the first attempt to functionally classify macrophages, while discovering that M1 and M2 macrophages also exhibited distinct metabolic programs[107]. Later on, Alberto Mantovani and colleagues systematized the knowledge acquired on the variety of stimuli able to induce mouse and human macrophage differentiation, establishing a continuum spectrum of cytokines/chemokines and cell surface receptors between M1 and M2 functionally polarized states[89] (Figure 6).

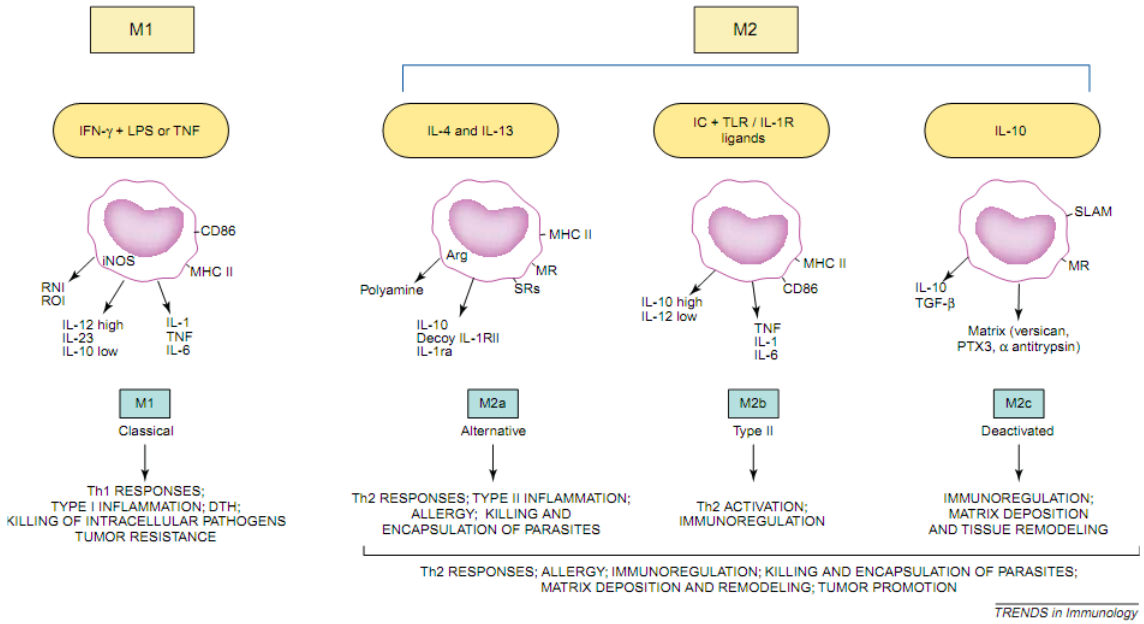

Figure 6 – Macrophages subsets described by Mantovani and colleagues in 2004. Abbreviations: DTH - delayed-type hypersensitivity; IC - immune complexes; IFN-γ - interferon-γ; iNOS - inducible nitric oxide synthase; LPS - lipopolysaccharide; MR - mannose receptor; PTX3 - the long pentraxin PTX3; RNI - reactive nitrogen intermediates; ROI - reactive oxygen intermediates; SLAM - signalling lymphocytic activation molecule; SRs - scavenger receptors; TLR - Toll-like receptor. Reprinted[89] with permission from Elsevier.

M1 macrophages were initially termed as classically-activated macrophages, as the term macrophage activation was first introduced some decades ago by Mackaness, upon observation of increased macrophage microbicidal activity during bacterial infection[108]. On their turn, M2 macrophages were designated by alternatively-activated macrophages, in opposition to the

classic manner. In summary, the M1 phenotype is characterized by the production of high levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12, IL-23, TNF-α, toxic intermediates, like NO and ROI, and high capacity to present antigens, upon exposure to IFN-γ in combination with LPS or TNF, being highly efficient at killing intracellular pathogens[89]. Upregulation of MHC class II, like HLA-DR, and of co-stimulatory signals, like CD80/CD86, is also characteristic of M1 macrophages.

On the other hand, M2 macrophages are involved in a variety of other functions like angiogenesis, tissue remodelling, wound-healing and anti-inflammatory processes. Due to their heterogeneity, M2 macrophages were categorized into three additional subtypes: M2a, induced by IL-4 or IL-13; M2b, induced by exposure to immune complexes (IC) and agonists of Toll-like receptors (TLRs) or IL-1R; and M2c, induced by IL-10 and glucocorticoid hormones[89]. With the exception of M2b subtype, M2 macrophages are low producers of pro-inflammatory cytokines, like TNF, IL-1 and IL-6. Metabolically, the arginase pathway is predominant in M2a and M2c polarized macrophages. Typical M2 markers include CD163, CCL18, arginase-1 and IL-10 (Figure 6).

The acquired knowledge about M1 and M2 macrophages was applied to several pathologic states, in an attempt to understand the macrophage functional phenotype associated with each disease. Generally, M1, also named pro-inflammatory macrophages, have been associated with autoimmune diseases, obesity and infectious diseases, while M2, also termed anti-inflammatory macrophages, have been associated with allergy, asthma and parasitic infections[89, 109]. In cancer, it is also accepted that M1 macrophages are involved in tumour suppression, while M2 are tumour promotors[62, 89].

Macrophage polarization: important considerations

Despite this apparent clear view of macrophage polarization status, the M1-M2 spectrum has raised many questions and some authors believe it has become too bipolar[110], while others argued that not every macrophage populations fit within the current classification[109]. This has led many to defend the existence of hybrid populations[111] or to suggest other phenotypes. For instance, the M3 macrophage "switch" phenotype was characterized by the macrophage reprogramming towards the M2 (anti-inflammatory) phenotype upon exposure to pro-inflammatory stimuli or, on the contrary, the acquisition of a M1 (pro-inflammatory) phenotype after exposure to anti-inflammatory stimuli[112].

As a consequence, other classification models have been proposed. In 2008, Mosser and Edwards suggested a new classification system integrating three fundamental macrophage functions: immune regulation (classically activated macrophages), wound healing (wound healing-associated

macrophages) and host defence (regulatory macrophages), which also considered the existence of intermediate groups with mixed phenotypes, as those observed in cancer and obesity[111]. Also the particular role of macrophages in atherosclerosis led to a new classification system of the subpopulations present in atherosclerotic plaques, either in mouse or human, that, besides M1 and M2 phenotypes, also included Mox, Mhem, M(Hb) and M4 ones, obtained respectively from macrophage stimulation with CXCL4, heme, haemoglobin/haptoglobin complex and oxidized phospholipids[113]. Other authors suggested a functional classification of macrophages according to wound-healing phases: early inflammatory phase (pro-inflammatory M1-like macrophages), late inflammatory phase (anti-inflammatory M2-like macrophages), granulation tissue formation/wound contraction (pro-fibrotic M2-like macrophages) and tissue remodelling (fibrolytic M2c/regulatory-like macrophages), as it apparently better resembles the *in vivo* complexity[114, 115]. In fact, although *in vitro* several functional macrophage profiles are able to be obtained, there is no sure to which extent they mimic the *in vivo* profile of macrophages, as tissues are very complex and dynamic environments exposing macrophages to a huge diversity of polarization stimuli[116].

Despite several efforts, all these alternative views of macrophage polarization were not yet completely accepted by the scientific community, remaining the continuum spectrum of M1-M2 macrophages as the main paradigm of macrophage activation. Nevertheless, it was recently recognized that the current macrophage polarization system needed to be revised, or at least updated, to overcome and also to clarify many concerns that have been raised in the last decade. These considerations were summarized in the following list, and should to be taken into account when interpreting data from macrophage polarization studies:

a) Different experimental conditions, namely macrophage source, initial cell seeding density, type of culture medium and tissue-culture conditions (particularly adherent surfaces) may lead to different macrophage activation status[110];

b) The type and concentration of stimuli used to induce similar phenotypes is highly variable, making difficult to compare data from different studies;

c) There is not a collection of markers clearly able to distinguish the different macrophage activation status. Therefore some considerations should be taken into account when selecting the best panel of macrophage markers:

- **i.** The same marker could be expressed by different macrophage phenotypes, differing only on the intensity level;
- **ii.** Activation marker expression could be temporal and spatially regulated[117];

iii. The use of activation markers should be complemented with the assessment of macrophage functions upon exposure to certain stimuli[117];
iv. Mouse and human macrophages exhibit some functional differences, what reflects the need for species-specific markers of macrophage activation;
v. Using a combination of markers is always the best option[110].

To uniform the experimental procedure of macrophage polarization induction and evaluation, a consensus document was published in 2014 by 25 authors, most of them well-known experts in the field of macrophage biology[67]. This publication summarizes important considerations, namely about the definition of the stimuli (termed the activator) as well as the collection of recommended markers used to induce or characterize each macrophage phenotype. For instance, authors suggested that in order to obtain both extremes of pro- and anti-inflammatory phenotypes, differentiated macrophages should be stimulated with IFN-γ or IL-4, respectively[67]. Importantly, they also reinforced that these phenotypes are two extremes of a continuous spectrum of activation and therefore macrophage stimulation with for example IL-10, LPS or a combination of LPS plus INF-γ give origin to intermediate phenotypes. The following figure summarizes the current knowledge about the several stimuli and markers used to promote or evaluate different functional phenotypes, either in human or mouse macrophages (Figure 7), obtained from the above-mentioned consensus document.

Man is not a mouse

Most of the current knowledge about macrophage biology, and specifically macrophage functional activation, come from murine models, raising two main concerns when transposing the acquired knowledge into the biology of human macrophages. First, the obtained results are not always concordant, even within macrophages from the same species. For example, macrophages from different mouse backgrounds exhibit distinct gene expression profiles upon exposure to the same stimulus, such as LPS[118]. Secondly, it is important to be aware that human and murine macrophages exhibit considerable differences, particularly regarding the production of iNOS/NO and arginases[62, 119]. NO is produced by iNOS, an oxidoreductase that catalyses the conversion of arginine and oxygen to NO and citrulline, playing an essential role in antimicrobial and anti-tumour activities in mouse macrophages[120]. Although human tissues seems to express iNOS, there are no strong evidences that human macrophage cell lines or human monocyte-derived macrophages express it[119, 121]. Contrarily to mouse macrophages, human macrophages also do not seem to express arginases, which are hydrolases that metabolize L-arginine to L-ornithine and urea[62]. Overall, more studies correlating mouse and human macrophage biology or even a more frequent

use of human macrophages as working models are required to better understand the human immunity and the validity of preclinical studies performed in mouse models[62].

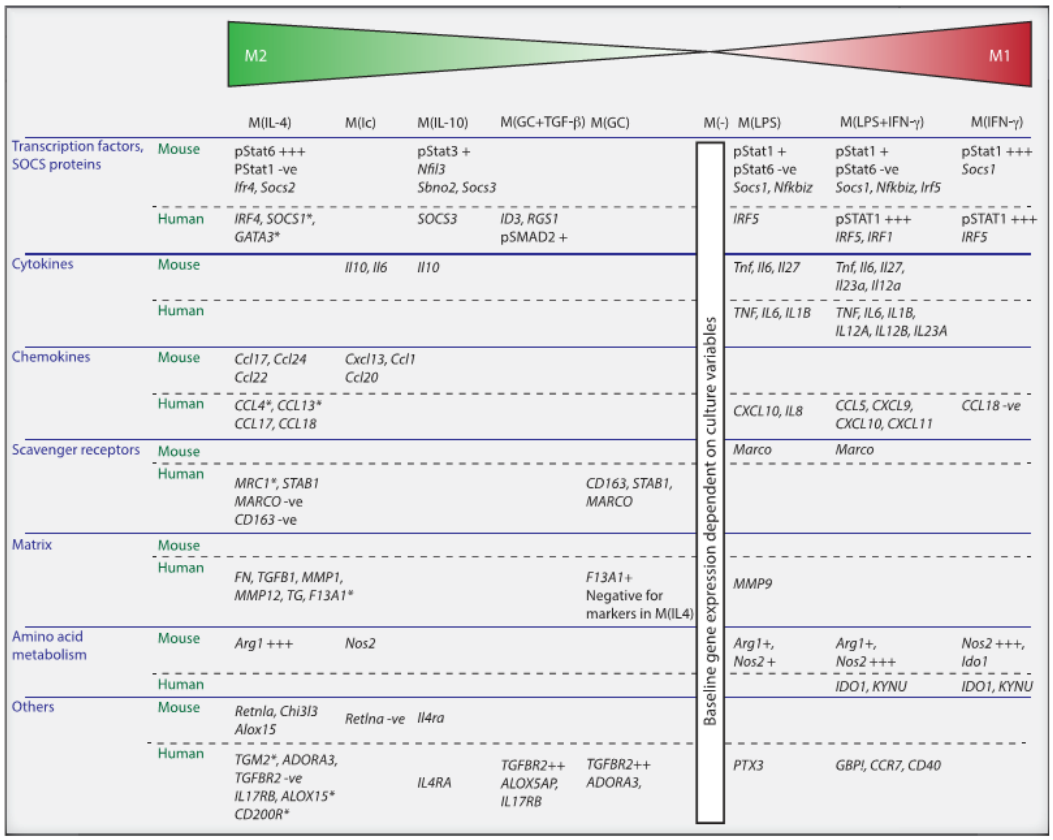

Figure 7 – Activation markers in mouse and human macrophages. Conventionally, macrophage polarization status is induced through stimulation with cytokines and/or growth factors and the obtained phenotype is usually confirmed by evaluation of the expression of several markers, which were here divided into several groups: transcription factors and suppressors of cytokine signalling (SOCS) proteins, cytokines, chemokines, scavenge receptors, matrix-associated and amino acid metabolism-related molecules, and an undefined group with a mixture of other markers. An asterisk indicates corroboration of human IL-4 genes by deep sequencing. Reprinted[67] with permission from Elsevier.

2.4 Tumour-associated macrophages (TAMs)

Similarly to tissue resident macrophages with inflammatory properties, TAMs are thought to have origin in peripheral-blood monocytes[66]. Although more details are still under investigation and TAM origin may differ according to the type of cancer, it is known that other factors besides CSF-1, may play a role on monocyte recruitment into tissues and on their differentiation into TAMs[122, 123, 124, 125]. In tumours, macrophages may exhibit pro- or anti-tumour properties, depending on the specific stimuli present at the tumour microenvironment[126] (Figure 8).

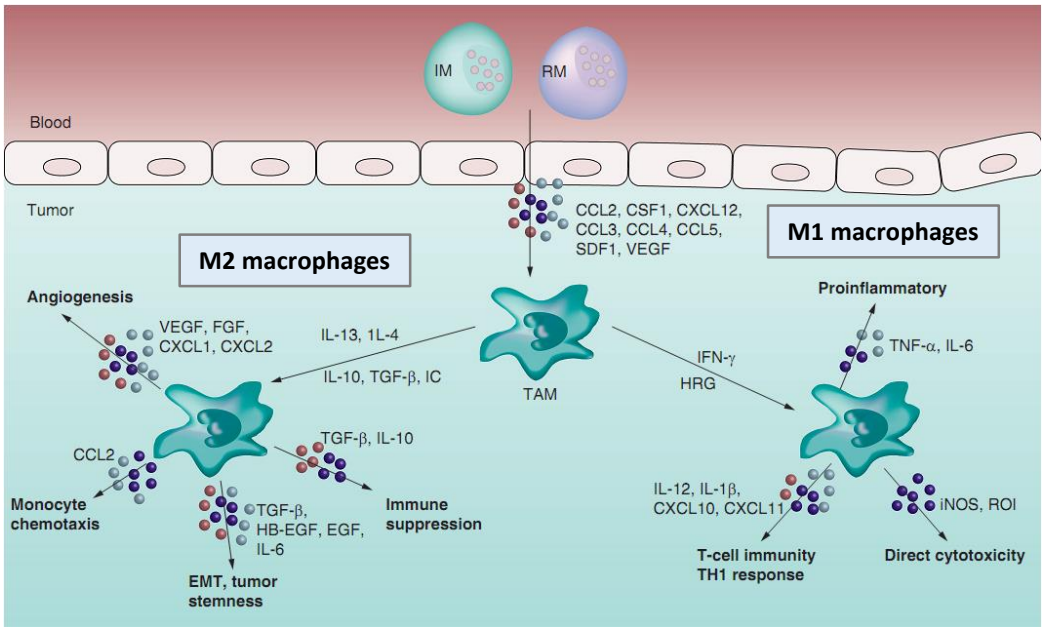

Figure 8 - Properties of tumour-associated macrophages. In tumours, M1 macrophages inhibit tumour growth through release of pro-inflammatory cytokines/chemokines, direct cytotoxicity or through promotion of T-cell immunity Th1 response, while M2 macrophages promote cancer cell invasion, angiogenesis, immune suppression, epithelial to mesenchymal transition (EMT) and tumour stemness, contributing to tumour progression. Abbreviations: HB-EGF - heparin-binding EGF-like growth factor; HRG – heregulin; IC - immune complex; IM - inflammatory monocyte; RM - resident monocyte. Adapted[126] with permission from Immunotherapy, as agreed by Future Medicine Ltd.

Tumour cell control by M1 macrophages

Pro-inflammatory and cytotoxic cytokines released by macrophages may act direct or indirectly, on tumour cells in order to kill them[126]. Macrophages are able to directly inhibit tumour progression, through at least three main mechanisms: i) inhibition of tumour cell division, ii) macrophage-mediated tumour cytotoxicity (MTC)[83] and iii) antibody-dependent cellular phagocytosis (ADCP)[127]. Inhibition of cell division by prostaglandins, IL-1 and TNF does not seem to require cell contact and occurs rapidly, acting on proliferating cells. On the other way, MTC requires cell contact, takes 1 to 3 days and is selective to tumour cells[128], leading to their lysis, through the action of TNF and of cytolytic proteases[83]. Ultimately, ADCP consists in macrophage ability to phagocyte monoclonal antibody-opsonized tumour cells, through engagement of the antibody Fc domain with the Fcγ receptors expressed by macrophages[127]. Indirect ways of macrophage-induced tumour cytotoxicity involves macrophage ability to process and present antigens to T cells and specific activation of Th17 cells, which produce IL-17 leading to neutrophil recruitment, or IL-12 production that can sustain Th1 and cytotoxic T-cell responses[126].

Role of M2 macrophages in tumour progression

During the earliest stages of cancer, macrophages are thought to present a pro-inflammatory M1-like phenotype, exhibiting a high capacity to present antigens and to produce many inflammatory mediators[89, 129]. Although some tumours present predominantly M1 macrophages associated with extended survival[136], immunohistochemistry analysis demonstrate that the predominance of this phenotype is less common[126]. In fact, as tumour grows and progresses, it seems that there is a switch into the M2 phenotype, which is followed by a gradual inhibition of NF-κB activity, through upregulation of the p50 NF-κB inhibitory homodimer[130, 131, 132]. This suggests that the key to understand the dual role of macrophages in tumours may not only rely on the recognized existence of subpopulations with pro- or anti-tumour functions, as well as on their presence at specific timings of tumour progression at specific geographic regions within the same tumour[117, 133]. Accordingly, both pro- and anti-inflammatory phenotypes may coexist within the same tumour[134, 135].

The notion that macrophages could have a preponderant role in tumour progression come from studies exploring CSF- 1 targeting and inactivation, which was reported to delay the development of invasive and metastatic mammary tumours[136]. Contrarily, CSF1 transgenic expression led to acceleration of later stages of carcinoma and significant increase in metastasis[136]. The ability of macrophages to promote tumour cell invasion, motility, intravasation, angiogenesis, extravasation and cancer cell growth at metastatic sites[137] will be exemplified herein.

i) Role of TAMs in cancer cell migration/invasion/intravasation

The first direct evidence for a synergistic interaction between macrophages and cancer cells during cell migration, invasion and intravasation *in vivo* came from experimental works published by Condeelis and Pollard, who were pioneers in understanding the role of macrophages in tumour progression, namely in breast cancer mouse models. They have first described the existence of a paracrine loop between macrophages and cancer cells, involving CSF1 (produced by cancer cells), EGF (produced by macrophages) and their receptors, favouring tumour cell migration and invasion[140, 141]. Later on, they also reported, using intravital imaging, that tumour cell intravasation occurred in association with perivascular macrophages in mammary tumors[138, 139, 140]. Briefly, the release of EGF by macrophages enhanced the invasion of EGFR-expressing cancer cells, which in turn secrete CSF1, which acts as a chemoattractant for CSF-1R-expressing macrophages[138, 139]. Other factors, like CXCL12 (SDF-1) and heregulin β1 (HRG-β1) (an EGF-like ligand), produced by different cells of the tumour microenvironment, also promote mammary tumour cell invasion, but always required the EGF/CSF-1 paracrine loop[141, 142, 143]. This suggests a crosstalk between stromal

and tumour cells and reinforces the important role of macrophages in mediating cancer cell migration and invasion. Notably, macrophage ability to increase tumour cell-ECM interactions, through production of osteonectin[144] or their proteolytic destruction of the matrix, particularly metalloproteinases[41, 145], facilitates cancer cell migration.

ii) Role of TAMs in tumour angiogenesis and at the metastatic site

Macrophage produces many angiogenesis-modulating factors, such as VEGF, bFGF, TNFα, IL-1β, CXCL8, COX-2, plasminogen activator, urokinase, PDGFβ, MMP-7, MMP-9 and MMP-12 (reviewed by Dirkx and colleagues[146]). Particularly, a macrophage population enriched in the expression of the angiopoietin 1 and 2 (ANG-1, ANG-2) receptor TIE2 seems to play an important role in angiogenesis promotion *in vivo*[147]. Hypoxia is also another major driver of angiogenesis and notably, in some studies, macrophages accumulate in hypoxic areas, which recruitment is mediated through VEGF[148]. In addition to the ability of macrophages to promote tumour angiogenesis, macrophages also help to prepare sites for metastatic cancer cells to seed, promoting cancer cell extravasation (by inducing vascular permeability through VEGF release), survival and persistent growth[40, 149]. A distinct macrophage population from the tissue resident one is suggested to be recruited at metastatic sites[149].

iii) Role of TAMs in tumour immune suppression

TAMs also secrete a wide range of molecules that, together with other immune cells, may negatively regulate anti-tumour responses: a) ligands for the inhibitory receptors programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4), PD-L1/PD-L2 and CD80/CD86 respectively, that upon activation supress the cytotoxic action of T cells, NK T cells and NK cells; b) ligands for death receptors FAS and TRAIL, which trigger caspase-dependent cell death in target cells; c) HLA-G and HLA-E that inhibits T cell function and NK cells, respectively; d) secretion of IL-10 and TGF-β that inhibit T cell effector functions and induce regulatory functions; e) secretion of CCL5, CCL20 and CCL22 that recruit Treg; f) secretion of arginase I, which by processing L-arginine compromises T cell function (Reviewed by Noy and Pollard[124]).

2.4.1 TAMs in colorectal cancer

In several types of cancer, the high density of TAMs, as detected by immunohistochemistry using lineage specific antibodies, was associated with bad poor clinical outcome. However, in CRC the presence of TAMs has been suggested as a good prognostic factor[116, 150]. In this particular microenvironment colonized by distinct microorganisms, the effect of TAMs was speculated to be

related with the release of pro-inflammatory cytokines, inhibition of cancer cell proliferation and recruitment of effector Th1 lymphocytes[151]. Additionally, some *in vitro* studies suggest that colorectal cancer cells may induce a mixed M1/M2 macrophage phenotype, which may depend on the molecular characteristics of the CRC cell line itself[152, 153]. Data regarding TAMs location also support the contradictory role of macrophage infiltration in CRC[154]. Some authors demonstrated a positive correlation between high macrophage infiltration at the tumour invasive margin and improved patient prognosis[155], while others demonstrated an association between high density of intratumoural TAMs count with the depth of invasion, the presence of lymph node metastasis and advanced tumour stage[156]. The enhanced expression, at the invasive front, of MMP-2 and -9 by immature myeloid cells was found to promote the invasion of CRC cells[157], and also enhanced macrophage MMP production, as well as macrophage ability to activate EGFR signalling pathway on cancer cells, contributed to the promotion of CRC cell invasion[103, 158].

The evaluation of the distinct macrophage subpopulations and of their distribution along tumour sections is extremely important to better understand the role of TAMs in cancer. However, obtaining reliable conclusions can be quite challenging, as different authors use different markers, cut-off definitions, outcomes, measurements, experimental procedures, and antibodies concentrations[150]. Importantly, several factors may contribute to misidentification of TAMs and their subtypes: i) the use of single-markers, which could be expressed by other cell types within the same tumour microenvironment, being not sufficient to clearly distinguish the distinct macrophage subpopulations[159]; ii) the lack of suitable markers for each macrophage species (murine versus human)[160]; and iii) the lack of representability of the analysed regions within the tumour. Therefore, more studies aiming to clarify the role of macrophages on colorectal cancer progression are imperatively required.

2.4.2 Targeting TAMs

TAMs have been considered interesting targets to fight against cancer, mainly due to their ability to promote tumour progression[61, 161] and to regulate tumour response to therapy, particularly radiotherapy, as will be further discussed[162]. However, additional reasons support this hypothesis, such as macrophage diploid nature, which enables phenotypic modulation, and their stable genome associated with low mutation rates, which suggests a low probability of developing a drug resistance profile[47, 163]. Targeting cells of the tumour microenvironment, like macrophages, allows modulating the activity of other important tumour counterparts with a relevant role in disease

progression[164]. Two major pathways for macrophage targeting have emerged, TAMs depletion and TAMs re-education[165] (Figure 9).

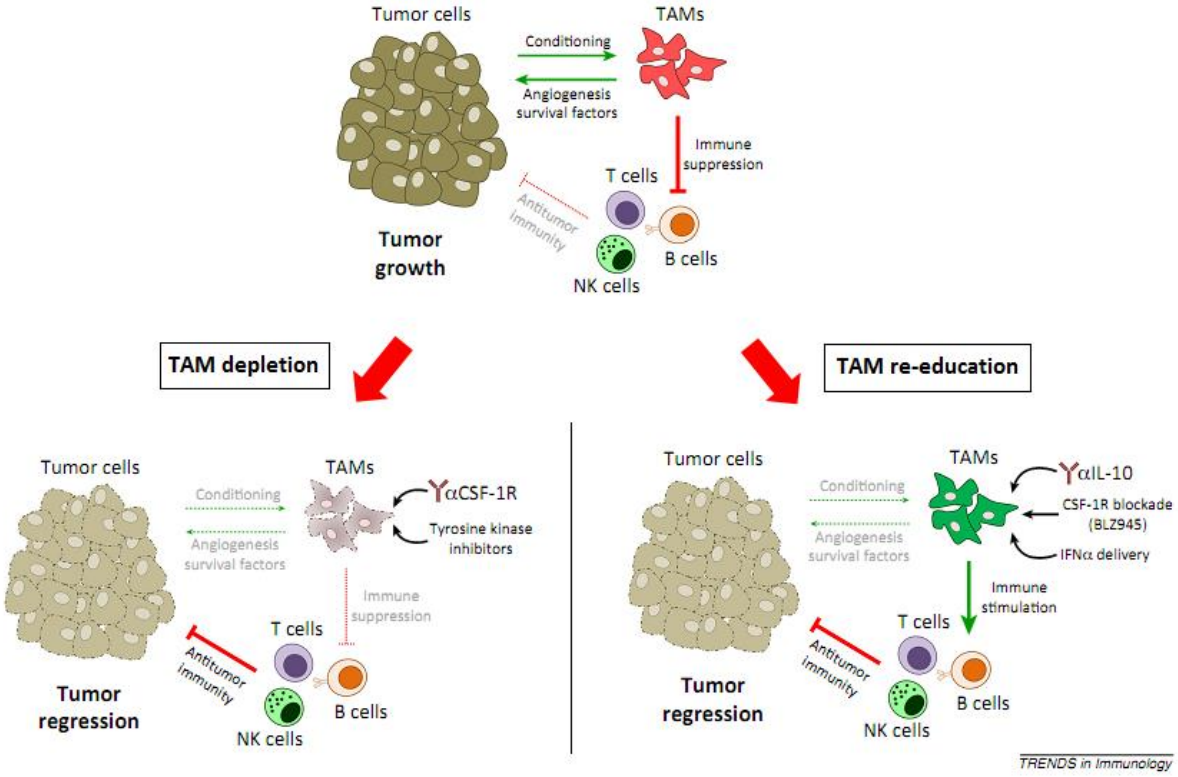

Figure 9 - TAMs depletion and TAMs re-education as two possible therapeutic strategies to target TAMs. Adapted[165] with permission from Elsevier.

The depletion strategy (*left side* of Figure 9) consists on the removal of TAMs from the microenvironment, achieved through inhibition of monocyte recruitment, monocyte differentiation into macrophages and TAMs survival[126, 165]. This strategy can be illustrated with the example of CCL2, a chemokine also known as monocyte chemoattractant protein-1 (MCP-1) expressed by tumour and stromal cells that recruits and modulates migration and infiltration of monocytes into the tumour. So, targeting CCL2-CCR2 axis, through specific CCR2 antagonist or knocking out host CCR2 expression, could lead to TAMs depletion[126, 166]. Also the use of CSF-1R antibodies or (quasi-)specific inhibitors of CSF-1R tyrosine kinase activity, like Sorafenib and Sunitinib, ablates any type of TAMs action over the tumour. Interfering with TAMs survival through the action of molecules that induce macrophage apoptosis, like bisphosphonates clodronate and zoledronic acid, is another possibility[167, 168, 169].

The re-education strategy (*right side* of Figure 9) is based on the principle that along tumour progression the local microenvironment may switch macrophages from M1 to M2 characteristics. This possibility offers a therapeutic option to functionally re-educate TAMs from a pro- (M2) to an

anti-tumour (M1) profile. TAMs re-education can be achieved through several approaches like IL-10 or CSF-1R blockade, exogenous administration of pro-inflammatory cytokines, or CD40 antibodies, which upregulate the MHC-II and the co-stimulatory molecule CD86 on TAMs surface, promoting T-lymphocytes activation and proliferation. Both TAMs depletion and TAMs re-education strategies culminate with blocking of immunosuppression by macrophages, which promotes T cells, B cells and NK cells cytotoxicity activity against tumour cells[165]. Some of these strategies, particularly the CSF-1R blocking, are currently under clinical trials (Reviewed by Panni and colleagues[126]).

Although TAMs targeting seems to be a good strategy to induce tumour regression, more research, namely on the modulation exerted by the tumour microenvironment on human macrophages, is required to clarify important issues[165]. How can the knowledge obtained from mouse models be translated into human macrophages? Is TAMs reprogramming a feasible approach? Could it overcome the activity of other immunosuppressive cells that normally complement TAMs functions? How long are these immunomodulatory strategies sustained?

3 Radiotherapy in cancer management

Since the last decade of the nineteenth century, soon after X-rays discovery, radiotherapy has been one of the most efficient and standard treatment options for a wide range of benign diseases, like skin conditions and hemangiomas, but mainly to fight against cancer[170, 171, 172]. Almost 50% of all cancer patients receives radiotherapy at some point of their treatment[173], although radiotherapy importance as a main treatment is highly dependent on the type of cancer. When early detected, lymphomas (Hodgkin and low grade Non-Hodgkin) and carcinomas of the skin (squamous and basal cell), prostate, lung (non-small cell), cervix, and head and neck, can be curable with radiation therapy alone[174]. However, the combination of radiotherapy with other modalities is required when the previous cancer types are diagnosed in more advanced stages or in other types of cancer, like breast, rectal and anal carcinomas, as well as in central nervous system and paediatric tumors[174].

Alone or in combination with surgery and/or chemotherapy, radiotherapy is mainly used with a curative intent when there is a probability of long-term survival after appropriate treatment. Exception for palliative radiotherapy, which aims to improve quality of life and to reduce symptoms when there is no hope of survival for extended periods, as it may occur in advanced or metastatic disease[175]. Contrarily to curative treatments, which last several weeks and are

generally delivered five days a week, in palliative ones radiation is delivered through fewer radiation exposures, but usually involving higher doses[176].

In general, radiation could be delivered from an external (external beam radiotherapy) or internal (brachytherapy) source or even systemically, through radioactive drugs[177]. In external beam radiotherapy, the tumour is irradiated with an external radiation beam, located at a certain distance from the patient, while in brachytherapy, sealed radioactive sources (like ^{192}Ir and ^{60}Co) are placed close to, or in contact with the target tissue. According to the international system of units (SI), the gray (Gy) is defined as the absorbed dose, which is equivalent to 1 J/Kg[178].

3.1 Types of radiation

According to the electromagnetic spectrum (Figure 10), radiation can be classified into two main categories: non-ionizing and ionizing, depending on its ability to ionize matter, i.e. to remove electrons from molecules[176]. Electric and magnetic fields, radio waves, microwaves, infrared, visible radiation and the lowest frequencies of ultraviolet are non-ionizing radiations, as they have insufficient energy to cause ionization. Regarding ionizing radiation, it is present in our lives either in a natural (cosmic rays, gamma rays from the Earth, radon decay products in the air and various radioactive isotopes found naturally in food and drink) or artificial (e.g. the use of X-rays in medicine) manner[179]. In medicine, ionizing radiation is used either for diagnosis (diagnostic radiology and nuclear medicine), recurring to lower doses of radiation, or for therapy purposes (radiotherapy) using higher doses[175, 179].

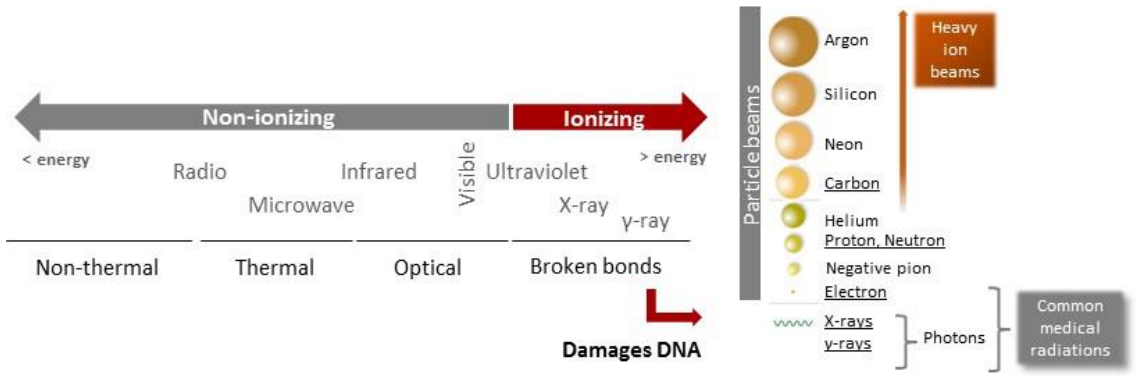

Figure 10 – Ionizing radiation and the most frequent beams used in radiotherapy. X-rays and γ-rays, globally referred as photon beams, together with electron beams are the most common medical radiations used. Compared with electrons, particles of the physical family of hadrons (protons, neutrons, pions and heavier ions) have greater mass, and their unique physical properties also confer some advantageous, although they are less frequently used in radiotherapy[176].

The ionizing radiation used in radiotherapy can be divided into two main groups: photon radiation (γ- and X-rays) and particle radiation (electrons, neutrons, heavy ions and α/β particles mainly

produced by radioactive substances)[180]. Particularly, external beam radiotherapy has evolved from low energy X-rays, through ^{60}Co γ-rays, to ever-increasing photon and electron energies, and ultimately to hadron therapy, which involves the use of protons, neutrons, and light and heavy ions, although it is still under clinical investigation[181].

To understand the main clinical applications of each type of radiation, it is important to elucidate some of their physical properties. Electrons primarily deliver their energy on the surface rather than going deeper into the tissues, being used for skin benign lesions, like keloids, which are characterized by dense fibrous tissue, as well as in intraoperative radiotherapy, namely in the treatment of initial-stage breast cancer[182, 183]. On their turn, photons deposit energy in tissues in a widely dispersed manner, being the peak of energy dose located relatively close to the surface, meaning that deep seated tumours can only be treated safely by focusing beams on the tumour from many angles[176]. Contrarily, other radiations induce a much localized pattern of energy along the trajectory of the particle, being the bulk of their energy located some centimetres in tissue depth, without delivering energy beyond that point, what significantly spares normal tissue[184, 185].

Photon X-ray is the most common type of ionizing radiation used in radiotherapy, being produced by linear accelerators widely available in hospitals where radiation treatment is provided. Contrarily, radiotherapy with high energy charged particles, essentially protons but also carbon ions, is only available at particular centres over the world, harbouring specific and very expensive infrastructures, most of them located at USA and Japan, with Germany leading the European centres[186]. Although still experimental, costly and logistically complex, high energy proton and heavy ion therapy promise higher tumour control rates with the exceptional advantage of sparing critical tissue. Therefore, they have been recommended for the management of childhood malignancies (protons), tumours near radiosensitive tissues or complex anatomical structures, and radioresistant or hypoxic tumours, which require a higher dose of radiation (neutrons/carbon ions)[181, 185].

3.2 Therapeutic ratio concept

The main goal of radiotherapy is to maximize the radiation dose to cancer cells, minimizing simultaneously the exposure of the surrounding normal tissues. Thus, in radiotherapy, the therapeutic ratio concept is defined as the optimal radiation dose capable of producing maximal probability of tumor control with minimal, or at least, reasonably acceptable damage to surrounding tissues[175] (Figure 11). Of note, very high doses not only enhance the probability of tumour cure but also increase the incidence of normal tissue damage, as a small volume of normal

tissue is unavoidably included in the radiation field. Additionally, the desirable therapeutic ratio shifts according the goal of the treatment, either radical, adjuvant or palliative and also with the tolerance of each tissue to radiation exposure[187].

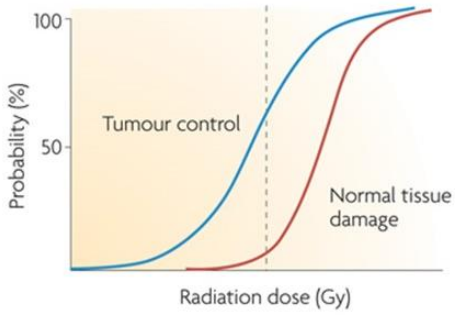

Figure 11 – Optimal dose-response curves for normal and tumour tissues in radiotherapy. The dotted line shows a theoretical dose associated with more than 60% of tumour control and about 5% incidence of normal tissue toxicity. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer][187], copyright (2009).

3.3 Principles of fractionated radiotherapy: the 5R´s

A better therapeutic ratio is normally achieved when radiation treatment is fractionated, i.e., when ionizing radiation is delivered to the patient over a period of weeks, rather than in a single session. The standard radiotherapy scheme involves the delivery of 2 Gy per fraction per day, over five days a week, during several weeks. However, other schemes make use of dose fractions larger than 2 Gy or smaller than 1.8-2.0 Gy, involving respectively fewer (hypofractionation) or more fractions (hyperfractionation) than the conventional scheme. The fractionation schedule used depends on the goal of the treatment and on the characteristics of the tumour. Briefly, hypofractionation modality decreases the opportunity for tumour cell regeneration during treatment, while hyperfractionation schemes (1.1-1.3 Gy/fraction) allow an increased tumour control, but lead to more severe early tissue reactions, due to increased total dose without overall time change[176].

In contrast to single dose radiotherapy, fractionated radiotherapy contributes to the probability of local tumour control and favours the survival of normal tissues over cancers, which could be explained by a group of factors, known as the 5Rs of radiobiology[176]. In 1975, Withers proposed the 4Rs of radiotherapy: recovery (from sublethal damage), cell-cycle redistribution, cellular repopulation and reoxygenation of the hypoxic portions of the tumour[188]. Some years later, Steel and colleagues added intrinsic cellular radiosensitivity as a fifth “R” to explain the different tolerance of tissues to fractionated radiation[189]. Lately, and although not yet assumed as news

"Rs", other important concepts like irradiated volume and restoration (long term recovery) came up[176]. An explanation of each of the 5Rs is provided below.

Recovery from sublethal damage (Repair)

The biological advantage of performing a fractionating radiation treatment rather than a single-dose delivery is mainly the recovery of healthy tissues from radiation-induced damage[176]. Ionizing radiation may cause lethal (fatal), sublethal or potentially lethal damage. The sublethal damage is the non-lethal cellular injury that can be repaired before the next fraction is delivered. Considerable recovery occurs within 15 min to 1 h, while complete recovery occurs within 6 h, but can be slower in some tissues, such as the spinal cord. Therefore, 8 h is considered the minimal interval between fractions. Contrarily to the majority of the tumours, normal tissues have intact repair pathways, thus more efficiently recovering from the sublethal damage before the next fraction[176].

Redistribution

Not every cells are at the same cell cycle stage when radiation is delivered. Cells that are in mitosis at the time of irradiation and cells that are in G2 and re-entering mitosis are the most radiosensitive. The surviving fraction at these times is less than a quarter of that seen for the most resistant cells, which are the ones in S phase (in particular the latter part of S phase). This resistance in S phase is probably due to homologous recombination, a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical DNA molecules and that occurs only during and shortly after DNA replication[176]. Therefore, to achieve an efficient killing of tumour cells by ionizing radiation, time between fractions is required for targeting surviving clonogenic cells which may be progressing from radioresistant S phase into a radiosensitive G2-M phase, a phenomenon termed redistribution, favoured by fractionated radiotherapy.

Repopulation

Repopulation concept describes the ability of survival clonogenic cells to repopulate the tumour after radiation and can be achieved through proliferation and/or reduced cell loss. Some tumours exhibit accelerated repopulation, what means that the clonogen doubling time during or shortly after irradiation exceeds the clonogen doubling time in untreated tumours. Thus, repopulation is an important factor in tumour resistance to radiotherapy and constitutes the rationale for accelerated fractionated regimens. On the other side, repopulation also describes the regeneration response of early-reacting tissues to fractionated radiation, which results in an increase in radiation tolerance with increasing overall treatment time[176].

Reoxygenation

The response of cells to ionizing radiation exposure is also dependent on the levels of oxygen present. Comparing the survival rate of mammalian cells cultured either under normoxic or hypoxic conditions, upon the same irradiation dose, we can observe an enhancement of radiation damage by oxygen[176]. Fractionating the dose allows then the reoxygenation of the hypoxic tumour regions (and hence radioresistant), which is required to more efficiently kill tumour cells.

Radiosensitivity

Cellular radiosensitivity is the sensitivity of cells to ionizing radiation *in vitro*. It is usually indicated by the surviving fraction at 2 Gy[176]. Radiation susceptibility is highly genetic-dependent, being deficiencies in DNA repair genes considered the main responsible for the clinical radiosensitive phenotype.

3.4 Radiotherapy for (colo)rectal cancer

Although radiotherapy is used as a palliative treatment in both colon and rectal cancers, particularly in those patients with inoperable disease or in whom comorbidity precludes operation, it assumes a more important role, together with other modalities, in rectal than in colon cancer. Therefore, the following guidelines will reflect the importance of radiotherapy in the management of rectal cancer.

Although surgery remains the mainstay of rectal treatment, adjuvant treatments, like radio- and chemotherapy, improve survival and reduce local recurrence by treating any residual microscopic disease[190]. Radiation could be delivered pre- (neoadjuvant) or post-operatively (adjuvant therapy). Pre-operative radiotherapy is considered more advantageous, improving local tumour control and treatment efficacy, by reducing tumour volume and consequently promoting tumour downstaging (Reviewed in Kye and Cho[191]). Radiobiological advantages are also inherent, as surgery-naïve tissues are better oxygenated. Importantly, pre-operative radiotherapy also preserves normal tissue functionality by: i) facilitating resection and enhanced sphincter-preservation after the reduction of tumour volume; ii) avoiding the occurrence of radiation-induced injury to the small bowel trapped in the pelvis, which can be postsurgical solved; and iii) allowing the surgical removal of the irradiated tissue (otherwise the anastomosis (surgical connection between two structures) could be affected by the consequences of radiation). Additionally, intensity-modulated and image-guided radiotherapy (IMRT-IGRT) have been used in the preoperative treatment of rectal cancer to reduce the irradiated volume of sensible organs, thereby decreasing normal tissue complications [192]. IMRT-IGRT combine the modulation of dose intensity within a given radiation

field together with the verification of the patient position and anatomy prior to treatment, through advanced treatment planning software and medical imaging.

The radiotherapy regimens currently available for rectal cancer treatment include short or long course pre-operative, or long course post-operative radiotherapy, as summarized in Table 2. Studies indicated that both short course and long course pre-operative regimens have similar efficacy, regarding survival and local recurrence[193]. However, the applicability of each one will depend on the tumour stage and patient performance status. For more details about the clinical protocol of rectal cancer management, please see the Annexes section.

Table 2 - Radiotherapy regimens currently available for rectal cancer treatment[190, 191, 194]. Low-dose chemotherapy is frequently delivered concurrently with radiation, particularly in long course pre-operative radiotherapy, in order to sensitize tumour to radiation, being thus termed as radiosensitizer chemotherapy[195]. Abbreviation: 5-FU - 5-fluorouracil, CT - chemotherapy, RT - radiotherapy. Note: resection margins are margins with apparently non-tumour tissue located around a tumour surgically removed, i.e. resected. R1 defines the microscopic margin limited by pathological examination, while R2 is the macroscopic margin frequently limited during the surgery with clips.

Regimen	RT schedule	Origin/Therapeutic overview
short course pre-operative RT (without CT)	5 Gy x 5 days - hypofractionated schedule	- developed in northern Europe (mainly in Sweden); - well-tolerated, practical and financially efficient; - generally followed by surgery 1 week later
long course pre-operative RT ± CT	1.8-2.0 Gy x 25-28 daily fractions + e.g. 5-FU	- developed in the United States and some European countries; - surgery performed 6 to 10 weeks after treatment;
long course post-operative RT ±CT	1.8-2.0 Gy x 25-28 daily fractions + e.g. 5-FU; Boost (R1/R2)	- induces additional morbidity; recommended when the risk of loco-regional recurrence is still high

Due to modern multidisciplinary treatment, local recurrence seems to be no longer the main problem in rectal cancer, contrarily to distant metastases, which constitute the main cause of treatment failure[196]. Although currently the choice of the type of radiotherapy regime is mostly based on clinical factors and anatomic imaging, more efforts are required to further individualize, or at least to stratify the treatment, and help to predict recurrence in rectal cancer patients[194]. Therefore, the future of rectal cancer treatment may rely on: i) the combination of advanced radiation strategies with novel radiosensitizers, aiming to improve cure rates and reducing short- and long-term toxicity[197] and recurrences; and also on ii) the discovery of predictive markers of pathological responses to therapy, which has been quite challenging, despite efforts from molecular biology[198, 199]. Currently, only *KRAS* mutant status predicts non-response to EGFR inhibition in metastatic disease[200].

To improve radiotherapy efficacy from a cellular and molecular point of view, researchers have at least two important tools available: rectal cancer cell lines, which are representative of primary rectal tumours[13] and may be useful to evaluate the efficacy of different radiosensitizers[201], and also histological tumour sections, obtained before (pre-treatment biopsies) and after radiotherapy (post-operative resections), although the small amount of tissue in tumour biopsy may restrict its analysis[202]. Regarding the first tool, it is important to remind that most rectal adenocarcinomas are characterised by chromosomal instability, frequently exhibiting mutated *APC* and inactivation of additional tumour suppressor genes in the P53 and TGFβ pathways, as well as activation of oncogenes, such as *KRAS* and *PI3CKA*, being *BRAF* mutations rare in rectal cancer[200]. Therefore, rectal cancer cell lines, particularly those, like SW1463[13], that exhibit most of these properties, are excellent *in vitro* models to better understand how response to radiation can be improved[201] (see Annexes). However, attention should also be paid to the interaction of cancer cells with other cell populations frequently present in the tumours, and which may contribute to cancer progression and response to therapy, as reviewed in Chapter 1.3.1 - *The tumour microenvironment modulates tumour progression*. Histological tumour sections are important tools to address this issue. Notably, the unique immunohistochemistry study aiming to address the role of macrophages as predictive markers of response to radiotherapy in rectal cancer patients demonstrated that irradiated tissue resections presented a significant reduction of HLA-DR positive (a pro-inflammatory marker) macrophages. Data also evidenced an association between a low score for HLA-DR positive macrophages and a better response to short-course radiotherapy, with up to 80% reduction in tumour cell density[203]. However, these preliminary results need to be consolidated by increasing the number of samples as well as the panel of macrophage markers used.

4 Molecular basis of cell response to ionizing radiation exposure

The effects of radiation exposure on biological systems generates processes that differ a lot in time-scale[176]. The physical phase consists of a series of ionization events, which occurs in less than 1 s, as a result of the interactions between radiation and the atoms that compose the tissues. Then, during the chemical phase, the damaged atoms and molecules react with other cellular components, leading to the formation of free radicals in less than 1 ms, and also to associated scavenging reactions. Finally, the biological phase starts with enzymatic reactions and repair processes, giving origin to the early, late or very late tissue effects, which may be observed for days, months or years after ionizing radiation exposure.

Ionizing radiation-induced cell damage can occur through direct contact of radiation beams with tissues they transverse, or indirectly through radiolysis of water, the major (80%) cellular component[204], with subsequent generation of reactive chemical species (ROS)[204]. In the presence of oxygen and at physiological pH, the major cellular reactive species include superoxide (O_2•−) and hydroxyl (•OH) radicals, and also hydrogen peroxide (H_2O_2)[204]. Additionally, ionizing radiation can also stimulate iNOS to produce large amounts of nitric oxide (NO), which upon reaction with oxygen generates reactive nitrogen species (RNS)[204].

4.1 DNA: the primary target

While most of the molecules in a cell (e.g. water, mRNA, proteins) have multiple copies being continually repaired and avoiding major damages, DNA molecule has only two copies, what limits its turnover, turning it the biggest target of ionizing radiation exposure[176]. Radiation induces a wide range of DNA lesions like: i) mutations; ii) base damage or loss; iii) cross-linking (DNA-DNA or DNA-protein) and iv) single- and double-strand breaks (SSBs and DSBs)[205]. As an example, 1 Gy of X-rays induces more than 1000 base damages, around 1000 SSB and about 20-40 DSBs[176]. Although mostly centred in DNA, ionizing radiation-induced damage also affects other macromolecules, namely lipids, resulting in lipid peroxidation and changes in membrane viscosity and dynamics, and proteins, by inducing aminoacid conversions, inter and intra-strand cross linking, cleavage, oxidation and carbonylation[206].

As the DNA is the primary target of radiation-induced lethality, the capacity of a cell to repair sublethal damage will depend on its ability to recognize it, via DNA damage sensor molecules, to transduce the damage signal, through expression and recruitment of specific proteins to damaged sites (DNA damage mediators), and finally, to activate repair mechanisms and induce cell cycle arrest (effector molecules)[207] (Figure 12).

DNA damage is detected by different protein complexes that recruit apical kinases like ATM (ataxia telangiectasia mutated, mainly activated by DNA DSBs[208]) and ATR (ATM Rad3-related protein, mainly activated by UV radiation and DNA replication stress[208]), which in turn phosphorylates and activates the histone variant H2AX at serine 139, named γH2AX. Within 5-30 min after its induction, DSBs and surrounded sites become phosphorylated by γH2AX, which is considered a sensitive marker of DNA damage and repair[209]. Then, propagation of DNA damage response occurs through two downstream kinases, the checkpoint kinase 2 (Chk2), mainly phosphorylated by ATM, and the checkpoint kinase 1 (Chk1), mainly phosphorylated by ATR, which in turn phosphorylate downstream effector molecules, such as p53 and cell division cycle (Cdc25). Almost every DBSs

are repaired within 2 h after radiation exposure, but the repair process could persist up to 24 h[210, 211]. The two major pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), involved in the repair of DNA DSBs were extensively reviewed elsewhere[212, 213, 214].

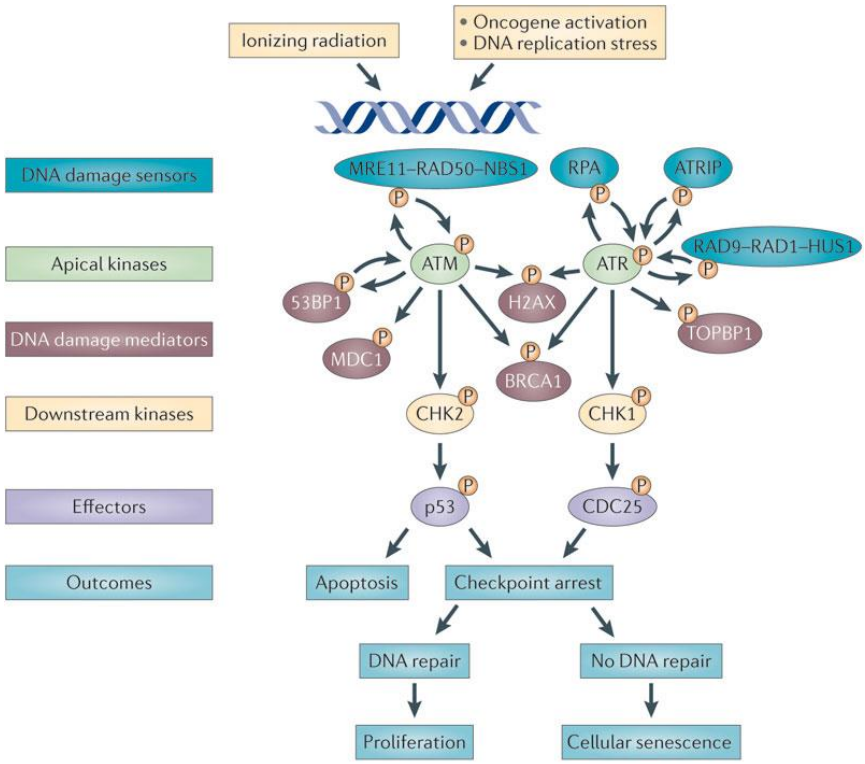

Figure 12 – The DNA damage response after induction of DNA single or double-strand breaks. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer][207], copyright (2012).

DNA damage sensor, mediator and effector molecules are all involved in DNA damage response (DDR), which activation may culminate with the induction of cell death, transient cell cycle arrest followed by repair of DNA damage and resumption of proliferation, or cellular senescence due to persistence of unrepaired DNA damage[207]. In irradiated cells, death through necrosis or apoptosis may be preceded by mitotic catastrophe, which is characterized by micronuclei formation as a result of incomplete DNA synthesis[215]. Particularly, fractionated radiotherapy was reported to induce apoptosis, through ROS generation and through caspase-3 activation and proteolytic degradation of one of its substrates – the poly ADP-ribose polymerase-1 (PARP)[216].

DNA damage in normal *vs* cancer cells

For more than a century, the rationale for using radiation against cancer cells relied on their high proliferative rate and defects in DNA repair machinery, what makes them more sensitive to radiation-induced DNA damage than normal cells and thus excellent targets for pharmacological modulation[19, 177, 217]. A brief explanation of this concept will be provided. In irradiated normal cells, radiation-induced p21 and Cdc25A activation, by the respective upstream targets p53 and Chk2,

leads to cell cycle arrest at G1/S phases and promotion of DNA repair, before entering into S phase, to avoid DNA damage transmission[218]. An arrest at G2/M may also occur through inhibition of Cdc25C by Chk2 or Chk1. However, in cancer cells, p53 is frequently mutated, blocking downstream signalling, thereby limiting the cell cycle arrest at G1/S, meaning that only intra-S and G2 checkpoints can be subjected to pharmacological inhibition[218]. The rationale for the use of cell cycle checkpoint inhibitors, together with genotoxic therapies like radio- and chemotherapy, is that this strategy will prevent cell cycle arrest as well as an effective initiation/completion of DNA repair after DNA damage[219]. In p53 pathway-proficient cells (e.g. normal cells), pharmacological inhibition of G2/M checkpoint render cells relatively radioresistant, as only those that already passed the active G1/S checkpoint are affected by inhibition of G2/M checkpoint[219]. On the other side, as p53 pathway-deficient cancer cells do not have a functional G1/S checkpoint arrest, they are more sensitive to inhibition of the remaining S and G2 checkpoints by G2/M checkpoint inhibitors, passing through mitotic phase without repairing DNA damage, which results in additional cytotoxicity that can easily lead to cell death[218, 219]. Besides checkpoint inhibitors, several other drugs aiming to inhibit ATM, ATR and also DNA-PKs (DNA-dependent protein kinase, another key molecule in the repair of DNA DSBs), have allowed to explore new radiosensitization strategies, being some them under preclinical studies[220].

4.2 Radiation-induced signalling pathways and development of radioresistance

Although water radiolysis-generated free radicals have a very short life span, persistent oxidative stress, which can last for several hours or days after radiation exposure, is likely to be generated by mitochondria[221]. ROS production and progressive damage induce lipid peroxidation and protein inactivation, with major consequences for signal transduction. Generally, sphingomyelinase pathway is associated with apoptosis induction, while other signalling pathways, like those mediated by receptor tyrosine kinase (RTK), such as MAPK and PI3K/AKT, or NF-κB, may be involved in cell survival upon irradiation, being frequently associated with cancer cell radioresistance (Figure 13), as will be here briefly detailed.

Sphingomyelinase pathway

At the plasma membrane, ionizing radiation activates, through a ROS/RNS-dependent mechanism, the membrane-bound acid sphingomyelinase (ASMase) enzyme, which converts sphingomyelin into ceramide, a simple sphingolipid[223]. Ceramide then acts as a secondary messenger in apoptosis

activation, inducing mitochondrial translocation of pro-apoptotic Bax and consequent cytochrome *c* release (Figure 13)[222, 223].

Concomitantly, ceramide was also described to activate cathepsin D[224], which in turn promotes apoptosis through cleavage of pro-apoptotic Bid[225]. Ceramide-mediated apoptosis seems to be particularly relevant for endothelial cells in response to radiation exposure[223, 226]. Increased ceramide content also promotes reorganization of the plasma membrane, ultimately leading to ceramide-enriched large platforms, which enable the clustering and effective signalling transmission of transmembrane receptors, like death (TRAIL, CD95, TNF), Toll-like receptors (TLR2, 4, 5) and cytokine receptors (IL-1R)[227, 228]. Contrarily to radiosensitive cells, which form these ceramide-enriched platforms upon radiation exposure, radioresistant cells are defective in the formation of these structural rearrangements, which is associated with their lack of ASMase activation[229].

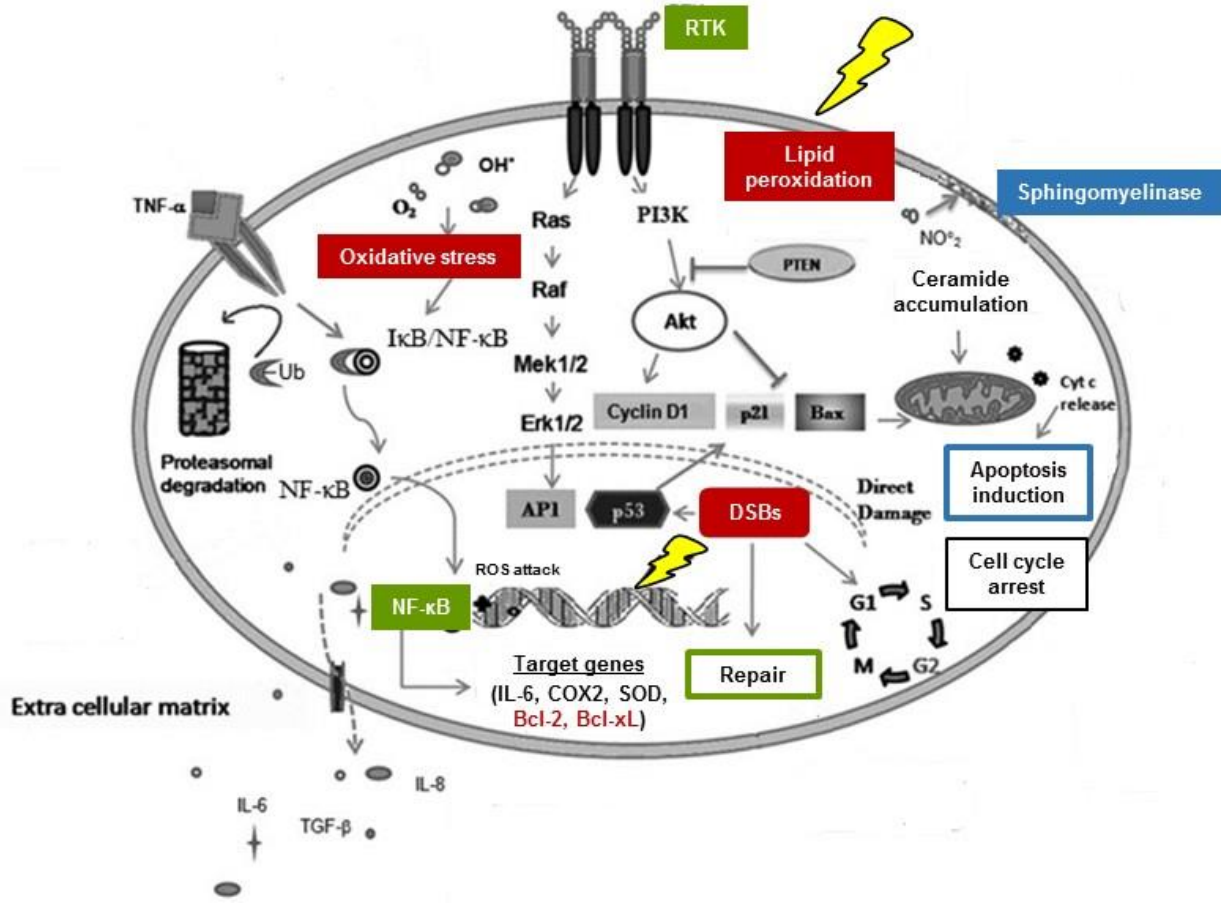

Figure 13 - Radiation-induced signalling pathways. Induction of DNA double-strand breaks (DSBs) and reactive oxygen species (ROS), which generates oxidative stress, are the primary effects (*red highlighted*) of radiation exposure. Activation of sphingomyelinase pathway is mainly involved in apoptosis induction (*blue highlighted*), while receptor tyrosine kinase (RTK) and NF-κB-mediated signalling pathways are mostly involved in cell survival and DNA repair pathways (*green highlighted*), upon radiation exposure. Adapted[206] with permission from Elsevier.

Receptor tyrosine kinase signalling

Receptor tyrosine kinases (RTKs), an important group of transmembrane receptors essentially activated by growth factors, are involved in a variety of cellular processes, as proliferation and differentiation, cell cycle control, cell survival and metabolism, and cell migration and invasion[230].

Among the 20 RTK subfamilies, the ErbB subfamily comprises four distinct members: ErbB-1, mostly recognized as epidermal growth factor receptor (EGFR), ErbB-2, also known as Her2 or Neu receptor, ErbB-3 and ErbB-4[231]. These receptors are ubiquitously expressed and activated by homo- or heterodimerization with receptors of the same or other subfamilies. Upon radiation exposure, the inhibition of protein tyrosine phosphatase function by increased ROS/RNS formation, maintains EGFR in a continuous activation state[231, 232]. Downstream EGFR, several signalling pathways important for cell survival, namely RAF-1-MAPK/ERK kinase(MEK)1/2-ERK1/2 and PI3K-AKT pathways, might then be activated (Figure 13). Notably, inhibition of EGFR or its downstream targets, like RAF and MEK, through antibody blockade or small-molecule inhibition, increased radiation cytotoxicity and enhanced cancer cell radiosensitization[231]. In addition, DNA repair may be also promoted by EGFR and its downstream effector ERK, mainly through the activation of DNA repair molecules, such as ATM and DNA-PKs[231, 233]. On its turn, Akt activation may counteract apoptosis and promote survival, through activation of the NF-κB pathway, inhibition of the pro-apoptotic proteins Bim, Bad and Bax, and activation of the pro-survival proteins XIAP (X-linked inhibitor of apoptosis) and Mcl-1 (myeloid cell leukaemia 1). [234]

Ionizing radiation also induces the overexpression of other RTKs, such as c-Met (also known as hepatocyte growth factor receptor - HGFR), particularly via ATM and NF-κB, promoting cell survival, proliferation and invasion[235].

NF-κB pathway

NF-κB/Rel is a family of transcription factors composed by five subunits, RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), which act as regulators of immune and inflammatory processes in response to injury and infection[236]. Unless they are activated or released from their inhibitors, called IκBs, NF-κB family members are kept in the cytoplasm as inactive dimers. Different pathways can lead to activation of NF-κB, but the classical (or canonical) one is the best characterized (Figure 14). The canonical activation of NF-κB is induced by antigen receptors, TLRs, cytokine receptors (as TNFR) and primarily activates the IκB kinase (IKK) complex (consisting of IKKα, IKKβ, and NF-κB essential modulator (NEMO))[237]. This results in the phosphorylation of IκBα, leading to its ubiquitination and subsequent proteasome degradation, releasing NF-κB complex containing p50-RelA, which then translocates to the nucleus.

On the other hand, the non-canonical pathway, engaged by members of the TNF-like family of cytokines, requires activation of IKKα by the NF-κB-inducing kinase (NIK) leading to the activation of p52–RelB heterodimers[238].

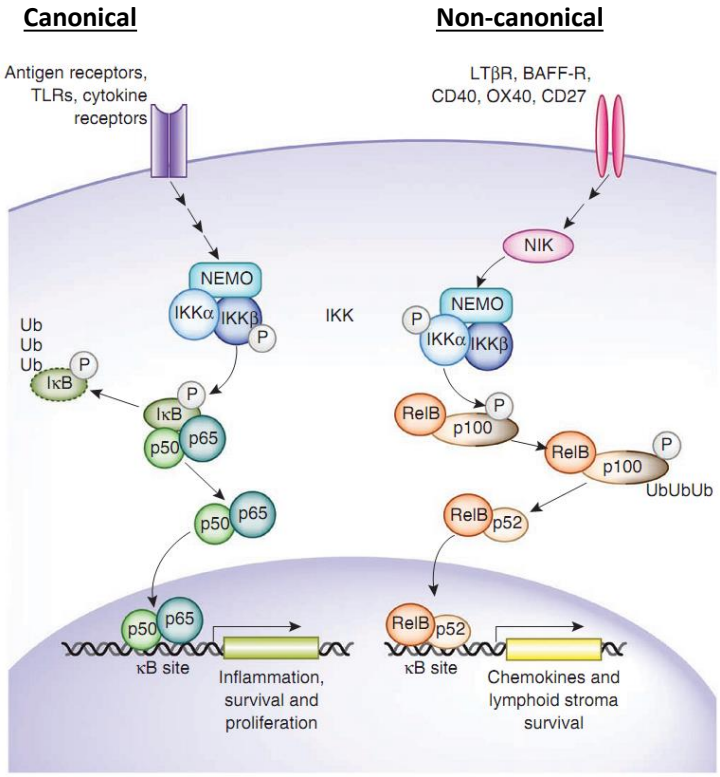

Figure 14 - The canonical and non-canonical NF-κB signal transduction pathway. This figure represents a very simplified overview of NF-κB signalling, as both canonical and non-canonical NF-κB pathways may interact with each other, thereby mutually influencing the transcription of NF-κB target genes[239]. Adapted by permission from Macmillan Publishers Ltd: [Nature][238], copyright (2013).

Overall, NF-κB is activated by a wide range of stimuli, like bacteria and their products (as LPS), inflammatory cytokines, physical and oxidative stress, environmental hazards, mitogens and growth factors (as M-CSF), being recognized as a central regulator of stress response[240]. As a genotoxic stress, ionizing radiation also causes NF-κB activation directly through response to DNA DSBs, involving ATM, which binds to NEMO, PIDD (p53-induced protein with a death domain) and DNA-PKs, or through ROS generation in the cytoplasm, which activate tyrosine kinases and NIK, leading to NF-kB nuclear translocation[241, 242]. Several NF-κB effector genes, namely anti-apoptotic (Bcl-2, Bcl-xL, XIAP and IAP - Inhibitor of Apoptosis Proteins), cell cycle regulators (cyclin D1) and the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) enzyme, which removes superoxide free radicals, may contribute to increased cancer cell survival upon irradiation and adaptive radioresistance[243].

Although NF-κB signalling inhibition has been appointed as a potential treatment adjuvant to radiotherapy[244], it does not always lead to cancer cell radiosensitization, and a better understanding of the heterogeneity of the cancer cell population, the level of NF-κB repression, the mechanisms underlying NF-κB inhibitors and the nature of the NF-κB subunits involved in radiation response, should be considered for successful results. An additional challenge is that NF-κB activation also induces the release of cytokines and chemokines (IL-1α, IL-1β, IL-6, TNF-α, CXCL8) by irradiated cells, modulating the activity of neighbour immune cells[245].

Metabolic alterations

Of particular importance for cancer cell response to radiation are radiation-induced metabolic alterations. A proteomic study addressing the response of mice to whole-body irradiation (20 Gy) demonstrated that the expression of proteins mainly involved in antioxidant response, energy metabolism, molecular chaperones and inflammatory response was altered by radiation exposure[246]. Additionally, the fact that mitochondria also contains DNA, which is subjected, upon radiation exposure, to the same chemical and structural modifications as nuclear DNA, although lacking an efficient DNA repair system, makes this organelle a particular radiation susceptible target[221]. Increased mitochondrial DNA copy number, which may contribute to higher mitochondrial mass, sequence variation, mutations and deletions may occur in the mitochondrial DNA, particularly in the 13 genes that code for subunits of the electron transport chain enzyme complexes and the ATP synthase (Reviewed in[221]). Among mitochondrial alterations, transient elevation of glycolysis has a preponderant role in response to radiation, facilitating DNA repair and contributing to cancer cell radioresistance[247, 248]. However, this effect seems to be dependent on the type of radiation. Notably, glycolysis enhancement, through increased LDHA, and upregulation of DNA repair networks were observed in HeLa cells after X-ray exposure, while carbon ions did not alter glycolysis but decreased amino acid metabolism and upregulated oxidative stress. This may justify the intense reduction of cell survival upon exposure to carbon ions than to X-rays[249]. Overall, effects of radiation on mitochondrial functional status are of major importance for cell response to radiation, particularly for the acquisition of a radioresistant phenotype.

4.2.1 Effect of radiation on non-cancer cells

The comprehension of the effect of radiation exposure on non-cancer cells has impact at two main levels: understanding i) how normal tissues respond to radiation, aiming to reduce its toxicity, and ii) how cells of the tumour microenvironment may contribute to cancer progression upon

irradiation. The second topic will be detailed in the Chapter 4.3, while the first one will be herein elucidated. The effect of radiation on endothelial cells, fibroblasts and immune cells will be addressed, since these are the main players of the cellular alterations induced by radiation exposure, namely vascular changes, fibrosis and inflammation processes, as a consequence of early or late biological effects that ultimately lead to acute and late adverse effects in normal tissues[250] (Figure 15).

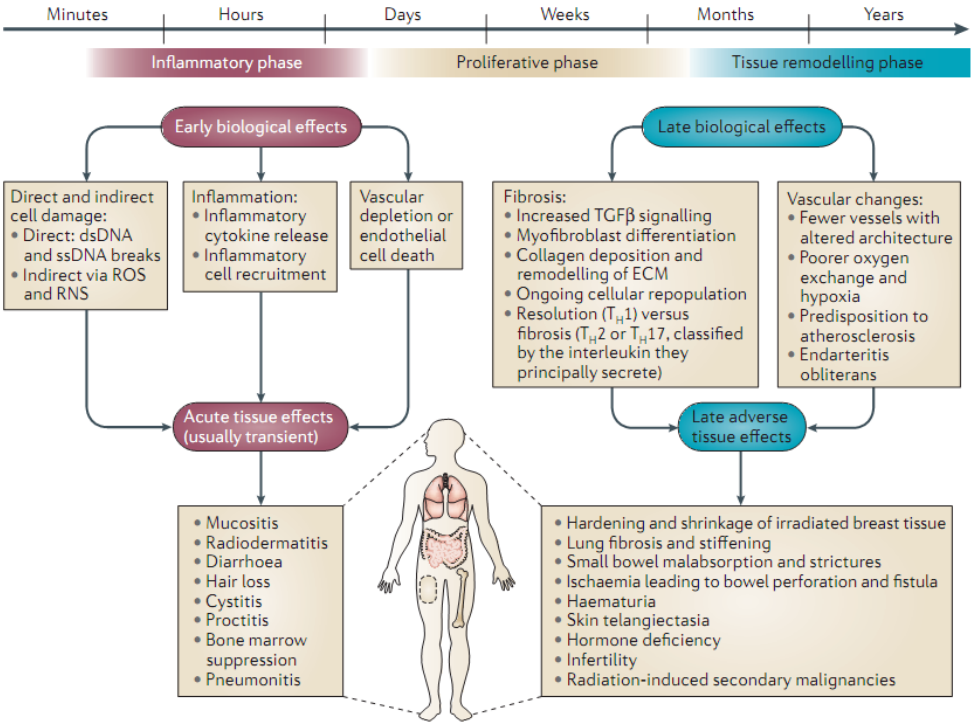

Figure 15 - Early and late cellular-mediated biological events after radiotherapy. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer][250], copyright (2015).

As an example of radiation-induced damage to normal tissue, in rectal cancer treatment progressive cell depletion and inflammation are responsible for radiation-induced early toxic effects, such as diarrhoea, cystitis, and perineal dermatitis, while vascular sclerosis and fibrosis are mainly involved in radiation-induced late effects, like bowel dysfunction, faecal incontinence, bleeding and perforation, genitourinary dysfunction, and pelvic fractures[251]. In summary, acute tissue effects, like mucositis and diarrhoea, occur during or shortly after completion of treatment, are usually reversible and generally not considered dose-limiting. Rapidly proliferating cells, at the skin, the gastrointestinal tract and the haematopoietic system are frequently the most affected tissues. On the other hand, late adverse tissue effects usually manifest six months after treatment initiation and may last for years. They usually occur in more slowly proliferating tissues, such as kidney, heart and central nervous system and include lung fibrosis, ischaemia, infertility, hormone deficiencies and second malignancies[187].

Endothelial cells

It is consistently described that radiation progressively reduces endothelial cell viability and proliferation, in a dose and time-dependent manner, leading to apoptosis and decreasing the formation of *in vitro* capillary-like structures[252, 253]. In animal models, ionizing radiation doses less than 1 Gy promote angiogenesis, suggesting that they can be used as an adjuvant therapeutic in the treatment of ischaemic patients[254], although these doses also promote tumour growth and metastasis in a VEGFR-dependent manner[255]. These results are supported by enhanced endothelial cell migration, VEGFR phosphorylation and hypoxia-induced VEFG expression after exposure to radiation doses up to 0.8 Gy, which do not affect endothelial cell proliferation and survival[255]. Although a single 5 Gy dose also reduces endothelial cell apoptosis[256], 20 Gy increases it and promotes the expression of angiostatic chemokines, as *CXCL9/10/11*[256]. Also the increased release of pro-inflammatory cytokines, such as IL-6 and IL-8, occurs in a dose and time-dependent manner upon endothelial cells irradiation[257]. Additionally, 3-5 days after exposure to 8 Gy most of the exponentially growing vascular endothelial cells exhibit a senescence-like phenotype[258]. This is accompanied by a significant reduction of cell cycle progression and DNA replication, as well as suppression of *in vitro* invasion and migration activities[258]. The radiation-induced endothelial cell apoptosis, which mainly involves the activation of the sphingomyelinase pathway[226], is a key event of both early (inflammatory) and delayed (fibroproliferative) radiation toxicity, particularly in the intestine[259, 260].

The radiation-induced vascular lesions mostly affect microvessels (capillaries, sinusoids), leading to capillary rupture or thrombosis, but has also negative consequences for medium-size vessels and arteries[261], with irradiated muscular arteries of radiotherapy patients exhibiting increased thickness[262]. This radiation sensitive phenotype of endothelial cells is particular relevant for normal tissue toxicity in dose hypofractionation schemes, as they involve the delivery of higher single doses per fraction[226].

Fibroblasts

Besides vascular changes, fibrosis is another common biological consequence of tissue exposure to ionizing radiation that can be sustained for years after therapy is completed. Fibrosis results from an increase in collagen fibres deposition and a reduction of fatty tissue, being clinically manifested as induration[176]. Among other cells, such as macrophages, fibroblasts constitute the main player of radiation-induced fibrosis[263]. Importantly, radiation-induced fibrosis is particularly different from wound healing fibrosis occurring in normal tissues repair[250]. In normal wound healing, a tissue injury is followed by infiltration of inflammatory cells, proliferation of epithelial

cells and tissue resolution through ECM remodelling[264]. However, in radiation-induced fibrosis an uncontrolled proliferative stage inhibits the resolution phase, resulting in late adverse effects and tissue morbidity. Upon radiation exposure, both ROS and NF-κB signalling activation may modulate radiation-induced fibrosis, particularly through the induction of pro-inflammatory molecules, namely TNF-α and fibrogenic cytokines, such as TGF-β[263]. TGF-β converts fibroblasts into matrix-producing fibroblasts, increasing the levels of collagen, particularly types I and III, fibronectin, but also of cytokines (such as HGF, TGF-β and CXCL12) and matrix remodelling enzymes (as MMPs)[263].

Immune cells

Initially, radiation may have an immunosuppressive effect caused by the destruction of the more radiosensitive immune cells. T helper cells, cytotoxic T cells, monocytes, neutrophils and, at a high degree, B cells, seem to exhibit a radiation sensitive phenotype when compared with macrophages, regulatory T cells, DCs and NK cells, which display a more radioresistant one[265]. Besides immunosuppression, ionizing radiation also induces pro-inflammatory processes. Increased production of cytokines, particularly IL-1, IL-6, TNF-α and TGF-β, occurs in irradiated tissues due to radiation-induced excessive ROS production, and may persist for long periods of time causing tissue injury[266]. Additionally, chemokines and their receptors, like CXCL12 and its receptor CXCR4, are involved in the recruitment of bone marrow-derived cells and of antigen-presenting cells, particularly macrophages and dendritic cells, into the irradiated tissues. Such recruitment may help to repair radiation-induced vascular damage, through the release of pro-angiogenic factors, or negatively contribute to tissue regeneration through increased production of pro-inflammatory cytokines[266]. Particularly macrophages are believed to be crucial for the transition between normal tissue damage and repair, due to their role in tissue homeostasis[267]. Irradiation may cause accumulation of lipid-containing macrophages, also known as foam cells, in the innermost layer of the medium-sized arteries, contributing to vascular obstruction and atherosclerosis-like lesions in several tissues[261].

4.2.1.1 Macrophage response to radiation

The first studies describing the macrophage response to radiation, dated from 50s-70s, were focused on the evaluation of macrophage phagocytic and immunogenic capacities in whole-body irradiated mice[268, 269, 270]. Later on, the introduction of macrophage *in vitro* analysis, even when extracted from whole body irradiated mouse, allowed the characterization of a variety of cellular aspects, more difficult to address *in vivo*. Macrophages *in vivo* irradiated seem to exhibit an activated phenotype, associated with increased DNA and RNA synthesis, enhanced choline uptake,

important for the synthesis of cell membrane phospholipid components, and increased levels of lysosomal enzymes, like acid phosphatase and cathepsin D[269]. Additionally, they increased *in vitro* phagocytic ability, as demonstrated by the percentage of macrophages ingesting fluorescent microspheres[271] and increased H_2O_2 production[272, 273]. The increased primed state for antibody-dependent cell-mediated cytoxicity (ADCC) was also observed, which contributed to the general observation that irradiated macrophages resembled IFN-γ stimulated macrophages[274, 275]. These studies clearly evidenced the acquisition of a macrophage activation status upon irradiation. Additionally, exposure of murine macrophages to 20 Gy (γ-ray) caused DNA synthesis blocking, without inducing a significant increase in cell death, maintaining their adherence and increasing lysosomal enzyme production, membrane ruffling, cell spreading, vacuolation and phagocytosis[273]. Protective agents, like SOD, catalase and iron-chelating agents, against radiation-induced free radicals, like H_2O_2, seemed to increase macrophage viability and phagocytic function upon irradiation[276].

In the last two decades, several reports evidenced that macrophage activation upon irradiation increased the production of pro-inflammatory mediators, such as IL-1β, TNF-α, NO, and was particularly enhanced when macrophages were exogenously stimulated with LPS or IFN-γ[277]. Particularly, NO production seems to be responsible for the enhanced *in vitro* cytotoxic activity of macrophages against tumour cells, after macrophage isolation from mice whole-body irradiated with low-doses[278]. Additionally, the involvement of NF-κB activation in radiation-induced NO production and iNOS expression in RAW264.7 mouse macrophages was demonstrated[279]. Accordingly, the nuclear translocation of NF-κB p65 subunit increased in peritoneal macrophages from whole-body irradiated mouse, evidencing increased secretion of pro-inflammatory IL-12 and IL-18 cytokines[280]. However, NF-κB nuclear translocation reduced when macrophage were irradiated with low doses (0.5-2 Gy). In a different context, a single 2 Gy dose seems to cause the induction of cytosolic Ca^{2+} rise, but with disruption of its oscillation, probably due to radiation-induced ROS, a mechanism that could be involved in radiation-induced lung toxicity[281]. Regarding macrophage morphology upon irradiation, a study with trout macrophages demonstrated that radiation increased the number of elongated cells, the size and number of filopodia/cell and F-actin reorganization[282]. Additionally, apoptosis was dramatically reduced 10 h after exposure to 5 Gy, while necrosis increased at earlier time-points, and efferocytosis reduced, but only after exposure to 0.5 Gy[282]. Finally, an interesting study comparing macrophages irradiated with X-rays or carbon-ions demonstrated that although metabolic activity was similarly affected by both types of radiation, carbon ions increased LPS-induced NO in a dose-dependent manner with a decrease in IL-1β production, and enhanced phagocytosis of latex beads up to 32 Gy [283].

Macrophage relative radiation resistant profile

Although a radiation resistant macrophage phenotype was suggested for decades[284, 285], the responsible mechanisms were only deeply explored in the past few years, using more advanced techniques, such as proteomic tools. In 2005, a first description of the proteome of primary bone marrow resident macrophages allowed to understand that their *in vivo* response to ionizing radiation (γ-rays, 0.5 Gy) was highly dependent on the genetic background of the mouse strains they were originated from[286]. Additionally, in 2007, the specific depletion of the pro-survival Mcl-1 was suggested as a possible mechanism responsible for sensitivity to radiation-induced apoptosis in mouse peritoneal resident macrophages, while Mcl-1 upregulation was observed in macrophages from radioresistant stains, which did not undergo apoptosis after irradiation (γ-rays, 40 Gy)[287]. In 2009, a second proteomic study demonstrated that RAW 264.7 macrophages overexpress the calcium regulatory protein calmodulin, after exposure to low-dose radiation (X- and γ-rays, 1 Gy), and suggested it could play a role in the promotion of DNA repair pathways involving H2AX phosphorylation[288]. In 2011, upregulation of some DNA repair proteins during monocyte maturation was suggested as a major protector against ROS, conferring radioresistance to differentiated macrophages, contrarily to their precursors[289].

The relevance of DNA repair for cell radioresistance seems to be transversal to several organisms. Studies on extreme radioresistant microorganisms demonstrated that protection of their proteome (from radiation-induced protein oxidation) and DNA, through intensive DNA repair mechanisms, were the main mechanisms involved in their extreme radioresistance[290]. It remains however to be determined the relevance of other signalling pathways for macrophage response to radiation, and whether human macrophages exhibit the same alterations observed in radioresistant mouse macrophages. Additionally, proteomic studies provide a valuable contribution to the investigation of cell radioresistance.

Overview of the current models to study macrophage response to radiation

Although a good amount of knowledge about macrophage response to radiation has been generated over the past years, the studies are highly diverse, namely regarding i) the type of macrophage model, ii) the dose and type of radiation used as well as the iii) the biological context. Most of the experimental works are still performed with mouse[291, 292] or rat[281, 293] macrophages, derived from cell lines[279, 283, 294] (mainly RAW264.7) or primary cultures[295, 296], and frequently stimulated with LPS, either pre-[279, 297] or post-irradiation[283, 298]. Thus, the lack of studies using human primary macrophages is evident. Regarding the use of radiation, the majority of the authors have investigated the effect of X-[280, 291] or γ-rays[279, 282], using high[281, 293, 299] or low[295, 297]

single doses, but rarely using fractionated schemes[281, 292], as it would be expected for the parallelism with the clinical context. Additionally, the whole-body[280, 291, 292] instead of local irradiation has been frequently applied in mouse models. However, this may originate misleading results, since in the clinics total body irradiation (TBI), either with photon or electron beams, is just delivered as part of a treatment regimen for bone-marrow transplantation[300] or to treat the rare skin disease mycosis fungoides[301], respectively, and not for solid tumours treatment.

Regarding the biological purpose, many of the studies investigate the role of macrophages upon irradiation in order to understand normal tissue reaction to radiation, such as lung toxicity and the acceleration of atherosclerosis development[302, 303]. Therefore, they use lower radiation doses than those delivered to tumours. Particularly, investigation on low doses has become a hot topic in radiation biology, mainly due to the potential increased risk of malignancy from exposure to low doses of radiation, as occurred with atomic bomb survivors. This data is of major health concern for workers of nuclear and medical industries, who are long-term exposed to low doses, and for the use of radiation for diagnostic purposes[304, 305]. Although the concept is frequently misused and not always clear, the UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) defined low doses as those equal or below 0.1 Gy[306]. Low dose radiotherapy (LD-RT) is used for benign painful and inflammatory diseases[296]. LD-RT generally involves the delivery of 0.3-1.0 Gy in 4-5 fractions for acute, and 1-3 fractions for chronic inflammatory disorders, corresponding to total doses of 3-5 Gy (acute) and 12 Gy (chronic)[307]. Radiobiology studies indicated that LD-RT promotes an anti-inflammatory, while high doses induce an inflammatory response (Reviewed by Rodel[307]).

4.3 The irradiated tumour microenvironment

The recognition that tumours are more than just agglomerates of cancer cells and that surrounding populations may promote cancer progression and contribute to the outcome of anti-cancer treatments, extended the study of radiation effects on cancer cells to other elements of the tumour microenvironment. However, communication within tumour microenvironment is complex and our knowledge on the effect of ionizing radiation on these elements and especially on the interaction that they establish with the existent cancer cells, is still limited. Briefly, the following major tumour microenvironment changes may be considered upon irradiation: i) decreased microvascular density (MVD), due to radiation-induced endothelial cell damage, ii) increased hypoxia, with aggregation of TAMs in hypoxic regions[308] and iii) promotion of intensive

immune responses, neither wholly immunostimulatory nor immunosuppressive, as will be further elucidated (Figure 16).

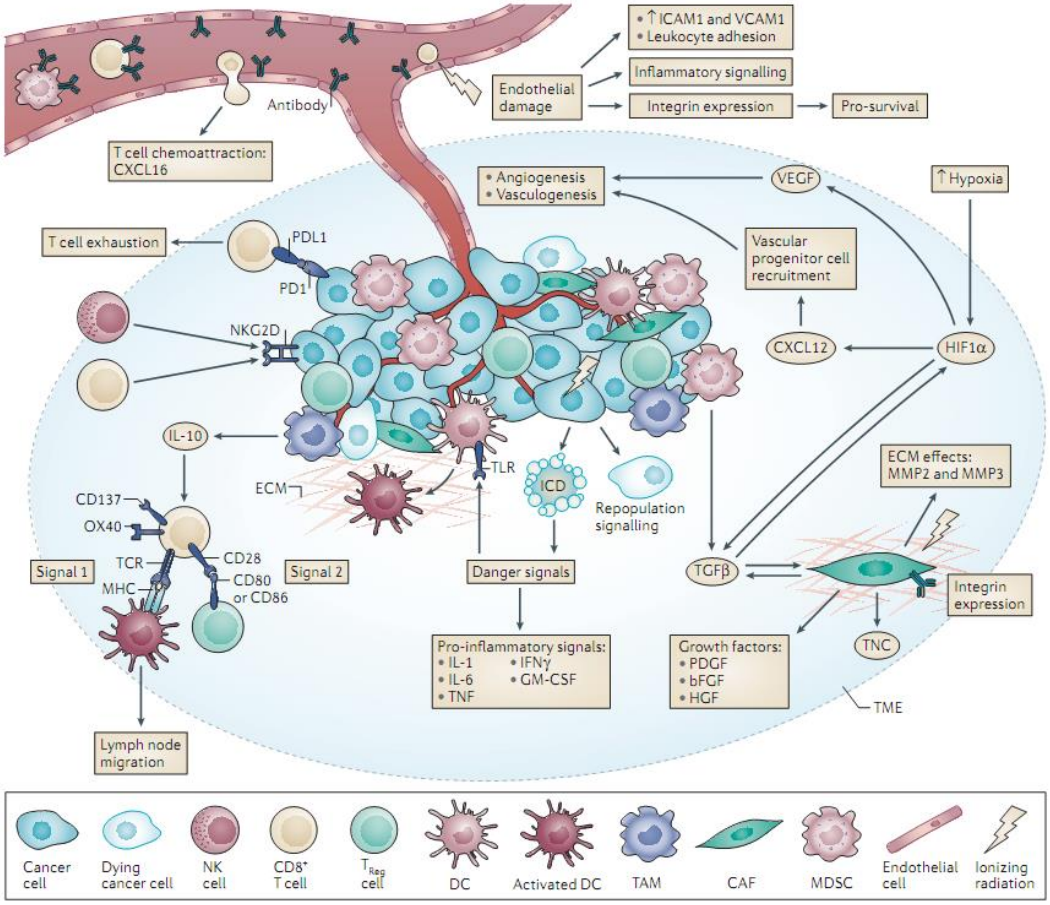

Figure 16 – Overview of the main radiation effects on tumour microenvironment cell populations. The death of radiation sensitive endothelial cells initiates the inflammation cascade, attracting more immune cells to irradiated tumour and potentiating the effects of hypoxia. Radiation-induced cancer-associated fibroblasts activation alters growth factor secretion, affecting ECM components production, and cytokine release. Within the immune compartment, radiation induces an increase of i) tumour cell antigen availability, ii) antigen processing and iii) pro-inflammatory signalling, resulting in DCs and T cell activation. Radiation-induced immune activation may however be inhibited by immune cell with immunosuppressive ability, such as Treg. Abbreviation: CTL4, cytotoxic T lymphocyte antigen 4; ICD, immunogenic cell death; MHC, major histocompatibility complex; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TCR, T cell receptor; TNC, tenascin C. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer][250], copyright (2015).

i) Decreased microvascular density (MVD)

In 2003, the work from Garcia-Barros and colleagues published in *Science*, demonstrated that not only tumour phenotype but also microvascular sensitivity could determine tumour response to radiotherapy[309]. Briefly, authors implanted cancer cells in an apoptosis-resistant acid sphingomyelinase (ASMase) knockout mice, which displayed reduced microvascular endothelial apoptosis and harbour faster growth tumour than those on wild-type microvasculature. Upon

exposure to single doses from 10 up to 20 Gy, tumours grown in knockout mice displayed reduced endothelial cell apoptosis and were more radiation resistant than the ones grown in wild-type animals. This study initiated a new molecular era in radiobiology and, together with previous works of Judah Folkman, supported tumour angiogenesis as a potential target of therapy[226]. As a consequence, endothelial cells and tumour vasculature became the best studied tumour microenvironment component regarding radiation-induced effects[250].

The elevated proliferation rate of tumour endothelial cells, which is 20-2000 times higher than that observed in normal tissues, increases their radiation susceptibility[310]. The reduction of microvascular density (MVD), mainly due to radiation-induced endothelial cell damage, increases inflammatory signalling and upregulates intercellular adhesion molecule-1 (ICAM-1) and VCAM-1, which attract innate immune cells[250]. On the other side, upregulation of integrin α3Vβ3 expression in irradiated endothelial cells increases their survival, mainly through AKT activation[250, 311]. In addition, pro-angiogenic growth factors, such as VEGF, PDGF and FGF, are overexpressed by irradiated cancer cells and activate the respective receptors on endothelial cells surface, counterbalancing their radiation-induced damage[312, 313].

ii) Increased hypoxia, with aggregation of TAMs in hypoxic regions

Vascular depletion also increases the effects of hypoxia, activating hypoxia-inducible factor 1α (HIF1-α) signalling and promoting angiogenesis and vasculogenesis through VEGF and CXCL12, respectively[250]. Cancer-associated fibroblasts also play a role in irradiated tumour microenvironment, not only through altered growth factor secretion and increased release of ECM modulators and cytokines, but particularly due to increased TGF-β production, which drives HIF-1α signalling[250].

Hypoxic chronic regions, which are located between normoxia and anoxic necrotic areas, are characteristic of many tumours and the association between low concentration of oxygen dissolved in tissues and irradiation was for long recognized as a limiting factor in radiotherapy[314]. Of particular concern for radiotherapy, and less well understood, is the acute/intermittent/cycling hypoxia, characterized by alternate periods of acute hypoxia or reoxygenation that may occur within a frequency of minutes to hours and even days[315]. In fact, blood flow redistribution and decreased oxygen consumption were suggested to occur in irradiated tumours[316]. At 24h post-irradiation, it seems there is an increase of the median distance to the nearest perfused vessel, indicating that several vessels lost their functionality and that tumour oxygen and nutrients have to travel more to reach the tumour cells that surround a given blood vessel[316]. The phenomenon of cycling hypoxia may also help to justify why sometimes an increase in the number of hypoxic

cells after irradiation is not observed [317], as expected by increased HIF-1α levels, since *in vitro* they have to remain hypoxic for several hours to exhibit binding to the hypoxia marker drug (pimonidazole)[315]. Additionally, mice pre-exposed to intermittent hypoxia present reduced radiation-induced apoptosis in both vascular and tumour cell compartments[318]. Also of major concern is the fact that acute hypoxic cells seem to exhibit higher metastatic potential than chronic hypoxic ones [319].

Contrarily to non-irradiated tumours, in which TAMs location is highly dependent on the tumour model and on the tumour stroma, in irradiated tumours TAMs tend to aggregate in pimonidazole positive hypoxic regions with low MVD[308, 320]. Additionally, two different TAM populations were identified: $CD68^+$ TAMS, which were highly centred in hypoxic regions, and $F4/80^+$ TAMS that lined on the edge of hypoxic regions next to necrotic areas. However, the distinctive role of both populations and whether this TAMs distribution matches with chronic and acute hypoxic regions remains to be clarified. In addition, $CD68^+$ TAMs aggregating in hypoxic regions after radiation tend to express higher levels of the anti-inflammatory macrophage marker arginase-1 (Arg-1) and seem to be associated with tumour re-growth after irradiation, what supports their eventual M2 phenotype[308].

As tumour irradiation abrogates local angiogenesis, tumours have to rely on the vasculogenesis pathway to develop more vasculature and thereby to support tumour regrowth. After tumour irradiation, this can be partially achieved through increased influx of $CD11b^+$ myeloid cells, particularly macrophages, a process mediated by increased HIF-1α, which upregulates SDF-1, the main driver of the vasculogenesis pathway[162,321].

iii) Promotion of intensive immune responses

Immune cells play an important role in the activation of a specific cancer cell death, termed immunogenic cell death (ICD) or immunogenic apoptosis, induced by chemotherapeutic agents and radiotherapy[250, 322]. ICD is characterized by the release, by dying cancer cells or tumour microenvironment elements, of danger signals termed damage-associated molecular patterns (DAMP). It occurs due to increased cellular stress (ROS production, endoplasmic reticulum stress, autophagy induction, cellular disintegration) induced by ionizing radiation exposure[323]. DAMPs can be exposed on the cell surface (e.g. calreticulin), passively released (e.g. histones or high-mobility-group box 1 (HMGB1)) or actively secreted (e.g. ATP)[324]. Contrarily to non-immunogenic (physiological) cell death, the immunogenic one stimulates the immune system, either local or systemically[325]. DAMP signals are recognized by multiple pattern-recognition receptors (PRRs) in several innate immune cells. For instance, calreticulin activates phagocytosis in macrophages

through CD191, while HMGB1 activates TLR in DCs. Additionally, danger signals lead to increased production of pro-inflammatory molecules, such as IL-1, IL-6, TNF and IFN-γ, which also activate DCs. After maturation, DCs become effective antigen-presenting cells, engulfing dying cells and presenting tumour antigens to $CD8^+$ T cells (Reviewed in [250]).

Overall, patient's dying cancer cells can act as a vaccine through stimulation of a specific immune response against cancer cells, with particular relevance for the most radioresistant ones[323]. However, this important immunostimulatory profile may be counterbalanced with the recruitment of locally suppressive immune cells[326], like TAMs, MDSCs and Tregs due to increased expression of molecules like ICAM-1, VCAM-1 and E-selectin, produced by endothelial cells and others within the tumour microenvironment[250], which compromises the development of therapeutically effective anti-tumour immune responses. For instance, increased expression of CTL4 by Tregs competes with the co-stimulatory molecules CD80 and CD86 in DCs for the binding to CD28 on $CD8^+$ T cells, thereby inactivating the co-stimulatory signal and compromising an effective T cell activation[327]. In summary, immune responses at the irradiated tumour microenvironment are neither fully immunostimulatory nor immunosuppressive.

4.4 Effects on non-target cells: the radiation-induced bystander signalling

In the past 20 years, experimental evidences have demonstrated that the biological effects described in cells directly exposed to ionizing radiation can also be observed in non-irradiated ones of the same organism[328, 329, 330, 331]. These non-targeted effects originated from irradiated cells were termed radiation-induced bystander effects[332], being the induction of DNA damage, mutations, transformation and cell death in non-irradiated cells the most prominent examples[333].

In vitro, the bystander effect can be studied through several strategies, including co-culture of irradiated cells with non-irradiated ones[334] or exposure of non-irradiated cells to CM from irradiated ones[329]. In 2005, results from the irradiation of a three-dimensional human tissue model system clearly supported the existence of *in vivo* radiation-induced bystander effects[67]. The authors demonstrated that non-irradiated cells, distant up to 1 mm from irradiated ones, exhibited an increase of 1.7-fold for micronuclei formation and 2.8-fold for apoptosis induction[332, 335]. Besides genetic instability, bystander signals may also induce protective responses involving terminal differentiation, apoptosis (removal of damaged cells) and radioadaptive responses[336, 337, 338, 339].

It is also important to note that not every cells emit or are sensitive to bystander signals, being the degree of response dependent on cell type, individual variability and physical aspects like

radiation dose and quality[329, 336, 340]. Regarding the dose, it is not yet clear whether bystander responses get saturated at relatively low doses (typically less than 1 Gy) or could also be relevant at higher doses[341]. However, it is already known that bystander signalling could be mediated through gap junctions and/or the release of soluble factors, like TNF-α and ROS, by irradiated cells, being immune cells, as macrophages, central players in the transmission of the radiation-induced tissue damage to non-irradiated cells[341]. This affects either adjacent or tissue distant non-irradiated cells, being the effect on last ones frequently referred as ionizing radiation-induced abscopal effects[336, 342]. The evidences in human body for abscopal effects remain scarce but may refer for example to the communication of the irradiated tumour with the respective metastasis[342].

Curiously, the type of radiation used seems to influence the triggering of secondary bystander effects by macrophages[343]. Accordingly, Dong and colleagues showed that γ-rays, but not carbon ions, were able to activate macrophages, mainly through IL-1β release, upon contact with irradiated cancer cells, which then induced more intensive secondary injuries, namely micronucleus formation, to other cancer cells[343].

The discovery of bystander and abscopal effects is very interesting from a biological point of view, but also raises many still unanswered questions. For example, what are the implications of bystander/abscopal responses for the long term effects of exposure to ionizing radiation[340]? Should we start to incorporate information from bystander/abscopal effects into the calculations of normal tissue complication[341]? How can we modulate the bystander/abscopal signals produced by immune cells in order to control cancer as a systemic disease and promote radiotherapy efficacy[342]? Thus, more investigations are required in order to better understand this effect and its consequences from a clinical perspective.

5 The challenge of improving radiotherapy efficacy

Radiotherapy field has been subjected to a great evolution in the last decades. The precision of external photon beams has evolved through the implementation of new treatment modalities, like IMRT and IGRT[181]. Additionally, the development of proton and heavy ion therapy, although under clinical trials, have allowed to spare critical tissues and manage radioresistant tumours[181]. Imaging advances, like the development of positron emission tomography (PET) or functional magnetic resonance imaging (fMRI) have been of great importance to identify tumours and their metastases, although they are still not able to detect subclinical disease[181]. The relevance of technological advances in radiotherapy has been extensively reviewed elsewhere[181, 344].

Despite technological advances, radiotherapy still faces its main challenge - to manage and control the systemic disease. Some factors, like large tumour and/or advanced tumour stage, inexact knowledge of the extent of the tumour and/or the movement of the tumour or patient during the course of radiotherapy, may explain why some cancers still present low survival rates after radiotherapy[218]. However, none of these reasons totally explain why tumours, with apparently similar sizes, stages, grades and delivered doses, respond differently, with some recurring and others not. In addition to the increased precision and accuracy of the treatment, the improvement of direct cancer cell killing and the reduction of normal tissue toxicity, the modulation of the tumour microenvironment seems another very promising strategy to improve the efficacy of radiotherapy. The future of radiation oncology may indeed rely on its fusion with biology[218].

5.1 Tumour microenvironment targets for radiosensitization

The idea that the tumour microenvironment elements could contribute to tumour progression and response to radiotherapy led to an intensification of the investigation of possible tumour microenvironment-based targets able to promote tumour radiosensitivity. This is of major importance as the current strategies coupled to radiation treatment involve the use of conventional chemotherapeutic agents, which are non-selective and induce significant normal tissue toxicity. Two major groups of targets for radiosensitization are enumerated, according to their biological effect, as reviewed in[250]:

i) **hypoxia targets**, which can be subdivided in those affecting endothelial cell survival (e.g. integrins), those that normalize the vasculature (TGFβ-R1, PDGF, PIGF, VEGFA and ANG2), those that prevent the vasculogenesis (CXCL12 (SDF-1) or CXCR4), those that alter oxygen delivery (e.g. through coupling accelerated radiotherapy with oxygen mimetic drugs, like carbogen and nicotinamide) and those that alter HIF signalling;

ii) immune targets, which can be subdivided into those that increase T cell numbers, those that affect T cell exhaustion or T cell checkpoint co-stimulation, and those that prime DCs, being the use of oncolytic viruses also an immunomodulatory stimulus.

Additionally, targets of fibrosis and cancer-associated fibroblasts may also be considered due to their negative effect in myofibroblast activation, TGF-β signalling, cancer-associated fibroblasts activation (PDGF, VEGF, bFGF, HGF) and ECM remodelling (e.g. MMPs, tenascin)[250]. Also combining radiation with VEGFR, PGFR and FGFR targeting promotes an anti-angiogenic effect through inhibition of PI3K/ATK signalling, which then induces apoptosis and causes tumour radiosensitization[345]. Particular inhibition of PDGF/PDGFR signalling may not only reduce tumour

angiogenesis but also radiation-induced fibrosis[312]. Inhibition of radiation-recruited $CD11b^+$ cells may therefore be blocked using antibodies or small molecules that inhibit HIF-1 or the interaction of SDF-1 with its receptors, constituting a new paradigm to improve local control by radiotherapy[346].

5.2 Combining radiotherapy with cancer immunotherapy

Cancer immunotherapy

Among the several elements within the tumour microenvironment, immune cells assume a preponderant role. Due to their ability to present different phenotypes after exposure to diverse environmental cues, immune cells have been investigated during the last three decades as possible targets for novel anti-cancer therapies[347]. Nowadays, the modulation of immune cell response for cancer treatment is no longer just an excellent idea, but also a clinical reality with very promising results[347].

The following scheme provides an overview of the current strategies being developed under the context of anti-cancer immunotherapy, including tumour-targeting and immunomodulatory monoclonal antibodies (mAbs); DC-, peptide- and DNA-based anticancer vaccines; oncolytic viruses; pattern recognition receptor (PRR) agonists; immunostimulatory cytokines; immunogenic cell death inducers; inhibitors of immunosuppressive metabolism; adoptive cell transfer; and others like macrophage-reprogramming agents (Figure 17) (Reviewed by Galluzzi and colleagues[347]) . Generally, some immunotherapy strategies specifically target one or more tumour-associated antigens, while others work by boosting natural or therapy-elicited anticancer immune responses in a relatively non-selective way[347]. Some modulatory strategies are still under investigation, but many others were already approved by the US Food and Drug Administration and the European Medicines Agency, for use in cancer patients[347]. Particularly checkpoint blocking antibodies (such as Pidilizumab and Ipilimumab), which are able to impair the function of receptors expressed on the surface of lymphocytes (like PD-1/CD279 and CTLA-4/CD152, respectively) or their corresponding ligands on tumour or other suppressive immune cells (PD-L1 and PD-L2), have shown exceptional efficacy in a wide range of malignancies, being already approved for the treatment of advanced melanoma, lung cancer, myeloma and metastatic renal cell carcinoma[348, 349].

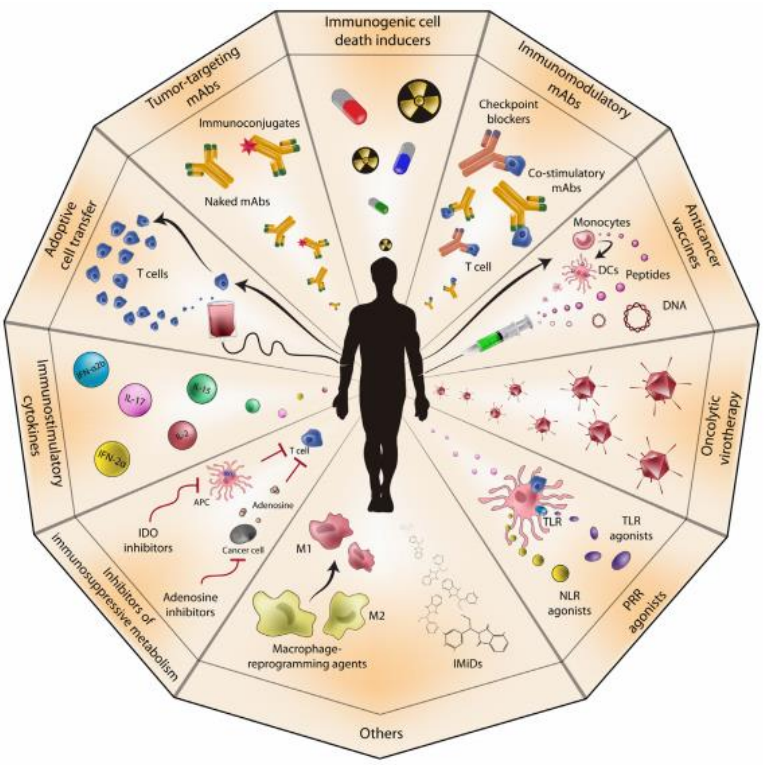

Figure 17 - Anticancer immunotherapy strategies. From the strategies depicted in the figure, only adoptive cell transfer, DNA-based vaccines and inhibitors of immunosuppressive metabolism are not yet licensed. Abbreviations: 1MT, 1-methyltryptophan; IDO, indoleamine 2,3-dioxigenase; IMiD, immunomodulatory drug; NLR, NOD-like receptor; pattern recognition receptor (PRR) agonists. Copyright[347] 2008-2015 Impact Journals, LCC. All rights reserved. Open-access license.

The combination with radiotherapy

Radiotherapy has an important role in selectively directing the immune response towards the tumour region. It promotes the recruitment of several immune cell populations into the tumour microenvironment, creating a unique opportunity to extend radiation-mediated damage to the tumour, improve clearance of residual cancer cells and also prime immune response to target distant disease[267]. So, combining both radio and immunotherapies seems to be a promising and efficient anti-cancer strategy. The irradiated tumour could then be transformed into an *in situ*, individualized vaccine[350]. Several studies either in preclinical or clinical phases are currently investigating the role of adequate fractionation of radiotherapy as well as timing of immunomodulation in glioma, breast, prostate, malignant melanoma and renal cancers, with already some reports of complete responses (Reviewed in[250]).

5.3 Targeting macrophages to decrease tumour radioresistance

As promoters of tumour progression and mediators of tumour response to therapy, macrophages are undoubtedly promising candidates to adjuvant immune strategies coupled with conventional

radiotherapy[161, 351]. Although most common immunotherapeutic strategies are essentially focused on the role of T cells on fighting against cancer cells and their antigens, macrophage-reprogramming agents have also a relevant role.

In order to develop macrophage-based tumour radiosensitizing strategies, the efficacy of TAMs targeting, either through macrophage ablation or modulation of their phenotype, should be evaluated under a context of tumour irradiation. Accordingly, general depletion of macrophages, through mice intraperitoneal or intratumoural injection of liposomal clodronate before irradiation, either delivered as a single (20 Gy) or fractionated dose (10 x 2 Gy), delayed tumour growth, enhancing the antitumour effects of ionizing radiation[352]. Additionally, targeting TNFα-induced macrophage VEGF production, using a soluble TNF receptor fusion protein, significantly decreased neovascularization and enhanced tumour radiosensitivity, upon exposure to 20 Gy[352]. Also inhibition of CSF-1 or SDF-1 signalling, through blockage of their receptors, before tumour irradiation, impaired monocyte recruitment and differentiation into TAMs, improving tumour response in gliobastoma[353, 354], prostate and other cancer models[355]. Regarding modulation of macrophage phenotype, the restoration of M1-like macrophages in tumour bearing mice upon injection of the glucose inhibitor 2-DG coupled with irradiation (10 Gy) correlated with a complete response[356]. The conversion of macrophages from the pro-tumour M2 phenotype to the anti-tumour M1 phenotype can also be performed using an *in vitro* IFN-γ-based delivery system, which decreased *in vitro* cancer cell invasion from 4 to 2 fold[357]. In fact, macrophage activation towards a cytotoxic M1 phenotype has been demonstrated to dramatically augment the effect of immunotherapy in murine models[358]. Therefore, drugs targeting macrophage pro-tumoural phenotype are of major importance. For instance, Trabectedin (alkylate DNA agent) acts at two main cell compartments: i) on cancer cells, inducing DNA breaks, promoting cell cycle arrest and growth inhibition and inducing cell differentiation; and selectively on ii) mononuclear phagocytes (monocytes and macrophages), inhibiting tumour growth and angiogenesis promoted by these cells, therefore modulating stroma-derived tumour resistance[359]. The use of Trabectedin was approved in 2007 for the treatment of soft tissue sarcoma and ovarian carcinoma[347]. Nevertheless, strategies to avoid TAMs differentiation into an M2 phenotype should be carefully conducted, because properties associated with this anti-inflammatory macrophage population, like tissue regeneration and matrix remodelling, crucial for normal tissue homeostasis, may be compromised[267].

Overall, combining radiation with modulation/targeting of macrophages seems a powerful radiosensitizing strategy. However more basic research involving the use of fractionated schemes mimicking closely cancer patients´ treatment, is still required.

Summary of key points

- Genetic alterations are one of the key features of cancer cells, particularly in CRC, conferring them growth advantage and resistance to therapy. CRC cell lines are excellent working models, representative of those features;
- Besides *genome instability & mutation*, other capabilities designated as hallmarks of cancer contribute to cancer cell survival, proliferation and dissemination. Most of these hallmarks are supported by the tumour microenvironment, which is a complex ecosystem composed by cancer cells, cancer-associated host cells and ECM, which all together may contribute to tumour progression;
- Particular attention has been given to infiltrating immune cells due to their dual role in promoting or blocking tumour progression. Among immune cells, particular interest was given to macrophages;
- Macrophages contribute to tissue homeostasis through immune surveillance activities, including phagocytosis (and efferocytosis), chemotaxis, antigen processing and presentation, and secretion of a wide range of molecules. Macrophage may be classified according to their functional status in a continuous spectrum between two extremes, M1 (pro-) and M2 (anti-inflammatory macrophages), although this classification is still a system under construction;
- In many solid tumours, macrophages are the most common leukocyte infiltrate, contributing to tumour progression through promotion of cancer cell migration/invasion/intravasation, angiogenesis and metastasis. Notably, they are also able to suppress tumour progression, by promoting direct or indirect tumour cell death. Therefore, macrophages constitute optimal targets to improve anti-cancer therapies;
- Radiotherapy is a widely common anti-cancer therapy, usually delivered in fractionated schemes instead of single doses, mainly to protect normal tissue from radiation-induced toxicity. Radiotherapy has a preponderant role in rectal cancer management;
- Although DNA is the main target of radiation-induced damage, other molecules are also affected, which results in stress signalling pathways activation. Additionally, the study of how tumour microenvironment populations contribute to treatment outcome may help to develop more efficient strategies against cancer cell radioresistance;
- The study of macrophage response to radiation mostly relies on murine models, single doses and whole-body irradiation, which do not mimic the clinical situation nor the biology of human macrophages. Comprehension of macrophage radioresistance and how this may contribute to tumour radioresistance is of major importance. In fact, macrophages seem to be involved in vascular recovery, which contributes to tumour regrowth after irradiation;
- Targeting tumour microenvironment populations, particularly immune cells, have a great potential to improve radiotherapy efficacy. Combination of radio- and immunotherapies is already in clinical trials and in the near future macrophage targeting could also be a valuable clinical option.

References

1. Ferlay J *et al.* GLOBOCAN 2012 v1.0. Cancer Incidence and Mortality Worldwide 2013 [cited 2015 17 Dec]; IARC CancerBase No. 11:[Available from: http://globocan.iarc.fr
2. World Health Organization. The top 10 causes of death. 2012 [cited 2015 17 Dec]Available from: http://www.who.int/mediacentre/factsheets/fs310/en/index2.html
3. American Society of Clinical Oncology. Major Milestones Against Cancer. [cited 2015 17 Dec]Available from: http://www.cancerprogress.net/timeline/major-milestones-against-cancer
4. American Society of Clinical Oncology. Colorectal Cancer. [cited 2015 17 Dec]Available from: http://www.cancerprogress.net/timeline/colorectal-cancer
5. Walther, A. *et al.* Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer* **9**, 489-499 (2009).
6. Pritchard, C.C. & Grady, W.M. Colorectal cancer molecular biology moves into clinical practice. *Gut* **60**, 116-129 (2011).
7. Vogelstein, B. *et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
8. Kitisin, K. & Mishra, L. Molecular biology of colorectal cancer: new targets. *Semin Oncol* **33**, S14-23 (2006).
9. Li, G.M. Mechanisms and functions of DNA mismatch repair. *Cell research* **18**, 85-98 (2008).
10. de la Chapelle, A. & Hampel, H. Clinical relevance of microsatellite instability in colorectal cancer. *J Clin Oncol* **28**, 3380-3387 (2010).
11. Davies, R.J., Miller, R. & Coleman, N. Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* **5**, 199-209 (2005).
12. Ahmed, D. *et al.* Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* **2**, e71 (2013).
13. Mouradov, D. *et al.* Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. *Cancer research* **74**, 3238-3247 (2014).
14. Sutton, P.A. *et al.* Predicting response to treatment for colorectal cancer: a review of relevant mechanisms and potential biomarkers. *Colorect. Cancer* **4**, 85-95 (2015).
15. Reimers, M.S., Zeestraten, E.C., Kuppen, P.J., Liefers, G.J. & van de Velde, C.J. Biomarkers in precision therapy in colorectal cancer. *Gastroenterology report* **1**, 166-183 (2013).
16. Papadopoulos, N. Clinical Value of Genomic Studies. In: Haigis, K.H. (ed). *Molecular Pathogenesis of Colorectal Cancer*. Springer: New York, 2013, p 258.
17. Huerta, S., Gao, X. & Saha, D. Mechanisms of resistance to ionizing radiation in rectal cancer. *Expert review of molecular diagnostics* **9**, 469-480 (2009).
18. Shin, J.S., Tut, T.G., Ho, V. & Lee, C.S. Predictive markers of radiotherapy-induced rectal cancer regression. *Journal of clinical pathology* **67**, 859-864 (2014).
19. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
20. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
21. Dhillon, A.S., Hagan, S., Rath, O. & Kolch, W. MAP kinase signalling pathways in cancer. *Oncogene* **26**, 3279-3290 (2007).
22. Yuan, T.L. & Cantley, L.C. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510 (2008).
23. Sherr, C.J. & McCormick, F. The RB and p53 pathways in cancer. *Cancer cell* **2**, 103-112 (2002).
24. Rivlin, N., Brosh, R., Oren, M. & Rotter, V. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes & cancer* **2**, 466-474 (2011).
25. Bellam, N. & Pasche, B. Tgf-beta signaling alterations and colon cancer. *Cancer treatment and research* **155**, 85-103 (2010).
26. Brech, A., Ahlquist, T., Lothe, R.A. & Stenmark, H. Autophagy in tumour suppression and promotion. *Molecular oncology* **3**, 366-375 (2009).
27. White, E. & DiPaola, R.S. The double-edged sword of autophagy modulation in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 5308-5316 (2009).
28. Grivennikov, S.I., Greten, F.R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883-899 (2010).
29. Blasco, M.A. Telomeres and human disease: ageing, cancer and beyond. *Nature reviews. Genetics* **6**, 611-622 (2005).
30. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-410 (2003).
31. Baluk, P., Hashizume, H. & McDonald, D.M. Cellular abnormalities of blood vessels as targets in cancer. *Current opinion in genetics & development* **15**, 102-111 (2005).
32. Friedl, P. & Alexander, S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* **147**, 992-1009 (2011).
33. Friedl, P. & Wolf, K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* **3**, 362-374 (2003).

34. Fidler, I.J. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* **3**, 453-458 (2003).
35. Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033 (2009).
36. Warburg, O. On respiratory impairment in cancer cells. *Science* **124**, 269-270 (1956).
37. DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G. & Thompson, C.B. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism* **7**, 11-20 (2008).
38. Jones, R.G. & Thompson, C.B. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes & development* **23**, 537-548 (2009).
39. Schuldt, A. Milestone 13: Environmental awareness (Nature Milestones in Cancer). *Nat. Rev. Cancer* **6**, S15 (2006).
40. Joyce, J.A. & Pollard, J.W. Microenvironmental regulation of metastasis. *Nat Rev Cancer* **9**, 239-252 (2009).
41. Lu, P., Weaver, V.M. & Werb, Z. The extracellular matrix: a dynamic niche in cancer progression. *The Journal of cell biology* **196**, 395-406 (2012).
42. Mareel, M., Oliveira, M.J. & Madani, I. Cancer invasion and metastasis: interacting ecosystems. *Virchows Archiv : an international journal of pathology* **454**, 599-622 (2009).
43. Hanahan, D. & Coussens, L.M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell* **21**, 309-322 (2012).
44. Pickup, M.W., Mouw, J.K. & Weaver, V.M. The extracellular matrix modulates the hallmarks of cancer. *EMBO reports* **15**, 1243-1253 (2014).
45. Paszek, M.J. *et al.* Tensional homeostasis and the malignant phenotype. *Cancer cell* **8**, 241-254 (2005).
46. Wozniak, M.A., Desai, R., Solski, P.A., Der, C.J. & Keely, P.J. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *The Journal of cell biology* **163**, 583-595 (2003).
47. Kitamura, T., Qian, B.Z. & Pollard, J.W. Immune cell promotion of metastasis. *Nature reviews. Immunology* **15**, 73-86 (2015).
48. Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* **357**, 539-545 (2001).
49. Heidland, A., Klassen, A., Rutkowski, P. & Bahner, U. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? *Journal of nephrology* **19 Suppl 10**, S102-109 (2006).
50. de Martel, C. *et al.* Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *The Lancet. Oncology* **13**, 607-615 (2012).
51. Hecht, S.S. Tobacco smoke carcinogens and lung cancer. *Journal of the National Cancer Institute* **91**, 1194-1210 (1999).
52. Arzumanyan, A., Reis, H.M. & Feitelson, M.A. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. *Nat Rev Cancer* **13**, 123-135 (2013).
53. Castle, P.E. *et al.* An association of cervical inflammation with high-grade cervical neoplasia in women infected with oncogenic human papillomavirus (HPV). *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **10**, 1021-1027 (2001).
54. Polk, D.B. & Peek, R.M., Jr. Helicobacter pylori: gastric cancer and beyond. *Nat Rev Cancer* **10**, 403-414 (2010).
55. Mattar, M.C., Lough, D., Pishvaian, M.J. & Charabaty, A. Current management of inflammatory bowel disease and colorectal cancer. *Gastrointestinal cancer research : GCR* **4**, 53-61 (2011).
56. American Cancer Society. Colorectal Cancer Facts & Figures 2011-2013. Atlanta: American Cancer Society; 2011.
57. Quail, D.F. & Joyce, J.A. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine* **19**, 1423-1437 (2013).
58. Lanca, T. & Silva-Santos, B. The split nature of tumor-infiltrating leukocytes: Implications for cancer surveillance and immunotherapy. *Oncoimmunology* **1**, 717-725 (2012).
59. Dranoff, G. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer* **4**, 11-22 (2004).
60. Balhara, J. & Gounni, A.S. The alveolar macrophages in asthma: a double-edged sword. *Mucosal immunology* **5**, 605-609 (2012).
61. Pollard, J.W. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* **4**, 71-78 (2004).
62. Murray, P.J. & Wynn, T.A. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology* **11**, 723-737 (2011).
63. Ginhoux, F. & Jung, S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature reviews. Immunology* **14**, 392-404 (2014).
64. van Furth, R. *et al.* The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World Health Organization* **46**, 845-852 (1972).

65. van Furth, R., Raeburn, J.A. & van Zwet, T.L. Characteristics of human mononuclear phagocytes. *Blood* **54**, 485-500 (1979).
66. Varol, C., Mildner, A. & Jung, S. Macrophages: development and tissue specialization. *Annual review of immunology* **33**, 643-675 (2015).
67. Murray, P.J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41**, 14-20 (2014).
68. Pixley, F.J. & Stanley, E.R. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends in cell biology* **14**, 628-638 (2004).
69. Stanley, E.R. *et al.* Biology and action of colony--stimulating factor-1. *Molecular reproduction and development* **46**, 4-10 (1997).
70. Hapel, A.J. & Stanley, R.E. Cytokines, Receptors and Signalling Pathways Involved in Macrophage and Dendritic Cell Development. *Eurekah Bioscience Database*. Austin (TA): Landes Bioscience; 2000.
71. Erblich, B., Zhu, L., Etgen, A.M., Dobrenis, K. & Pollard, J.W. Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. *PloS one* **6**, e26317 (2011).
72. Marks, S.C., Jr. & Lane, P.W. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. *The Journal of heredity* **67**, 11-18 (1976).
73. Wiktor-Jedrzejczak, W. *et al.* Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4828-4832 (1990).
74. Yoshida, H. *et al.* The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**, 442-444 (1990).
75. Hume, D.A. *et al.* The mononuclear phagocyte system revisited. *Journal of leukocyte biology* **72**, 621-627 (2002).
76. Yang, J., Zhang, L., Yu, C., Yang, X.F. & Wang, H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker research* **2**, 1 (2014).
77. Lorkowski, S. Monocytic and macrophage-like cell lines. 2008-2011 [cited 2016]Available from: http://www.infarktforschung.de/macrophages_cell_lines_uk.html
78. Tsuchiya, S. *et al.* Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International journal of cancer. Journal international du cancer* **26**, 171-176 (1980).
79. Auwerx, J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia* **47**, 22-31 (1991).
80. Tsuchiya, S. *et al.* Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer research* **42**, 1530-1536 (1982).
81. Kohro, T. *et al.* A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. *Journal of atherosclerosis and thrombosis* **11**, 88-97 (2004).
82. Daigneault, M., Preston, J.A., Marriott, H.M., Whyte, M.K. & Dockrell, D.H. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PloS one* **5**, e8668 (2010).
83. Burke, B. & Lewis, C.E. *The Macrophage*, 2 edn. Oxford University Press: New York, 2002.
84. Aderem, A. & Underhill, D.M. Mechanisms of phagocytosis in macrophages. *Annual review of immunology* **17**, 593-623 (1999).
85. Martin, C.J., Peters, K.N. & Behar, S.M. Macrophages clean up: efferocytosis and microbial control. *Current opinion in microbiology* **17**, 17-23 (2014).
86. Arango Duque, G. & Descoteaux, A. Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology* **5**, 491 (2014).
87. Friedl, P. & Weigelin, B. Interstitial leukocyte migration and immune function. *Nature immunology* **9**, 960-969 (2008).
88. Jones, G.E. Cellular signaling in macrophage migration and chemotaxis. *Journal of leukocyte biology* **68**, 593-602 (2000).
89. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends in immunology* **25**, 677-686 (2004).
90. Pixley, F.J. Macrophage Migration and Its Regulation by CSF-1. *International journal of cell biology* **2012**, 501962 (2012).
91. Janeway, C.A.J., Travers, P., Walport, M. & Shlomchik, M.J. Principles of innate and adaptive immunity. *Immunobiology: The Immune System in Health and Disease*, 5th edn. Garland Science: New York, 2001.
92. Itano, A.A. & Jenkins, M.K. Antigen presentation to naive CD4 T cells in the lymph node. *Nature immunology* **4**, 733-739 (2003).
93. Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M.K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **410**, 101-105 (2001).

94. Blum, J.S., Wearsch, P.A. & Cresswell, P. Pathways of antigen processing. *Annual review of immunology* **31**, 443-473 (2013).
95. Honey, K. & Rudensky, A.Y. Lysosomal cysteine proteases regulate antigen presentation. *Nature reviews. Immunology* **3**, 472-482 (2003).
96. Neefjes, J., Jongsma, M.L., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews. Immunology* **11**, 823-836 (2011).
97. Chen, L. & Flies, D.B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews. Immunology* **13**, 227-242 (2013).
98. Greaves, P. & Gribben, J.G. The role of B7 family molecules in hematologic malignancy. *Blood* **121**, 734-744 (2013).
99. Alberts B *et al.* Helper T Cells and Lymphocyte Activation. *Molecular Biology of the Cell*, 4th edn. Garland Science: New York, 2002, p 1616
100. Saftig, P. & Klumperman, J. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nature reviews. Molecular cell biology* **10**, 623-635 (2009).
101. Bewley, M.A. *et al.* A cardinal role for cathepsin d in co-ordinating the host-mediated apoptosis of macrophages and killing of pneumococci. *PLoS pathogens* **7**, e1001262 (2011).
102. Nathan, C.F. Secretory products of macrophages. *The Journal of clinical investigation* **79**, 319-326 (1987).
103. Cardoso, A.P. *et al.* Matrix metalloproteases as maestros for the dual role of LPS- and IL-10-stimulated macrophages in cancer cell behaviour. *BMC cancer* **15**, 456 (2015).
104. International Union of Immunological Societies/World Health Organization Subcommittee on chemokine, n. Chemokine/chemokine receptor nomenclature. *Journal of leukocyte biology* **70**, 465-466 (2001).
105. Gessani, S. & Belardelli, F. IFN-gamma expression in macrophages and its possible biological significance. *Cytokine & growth factor reviews* **9**, 117-123 (1998).
106. Schmieder, A., Michel, J., Schonhaar, K., Goerdt, S. & Schledzewski, K. Differentiation and gene expression profile of tumor-associated macrophages. *Seminars in cancer biology* **22**, 289-297 (2012).
107. Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J. & Hill, A.M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *Journal of immunology* **164**, 6166-6173 (2000).
108. Mackaness, G.B. Cellular resistance to infection. *The Journal of experimental medicine* **116**, 381-406 (1962).
109. Chavez-Galan, L., Olleros, M.L., Vesin, D. & Garcia, I. Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages. *Frontiers in immunology* **6**, 263 (2015).
110. Martinez, F.O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports* **6**, 13 (2014).
111. Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* **8**, 958-969 (2008).
112. Malyshev, I. & Malyshev, Y. Current Concept and Update of the Macrophage Plasticity Concept: Intracellular Mechanisms of Reprogramming and M3 Macrophage "Switch" Phenotype. *BioMed research international* **2015**, 341308 (2015).
113. Colin, S., Chinetti-Gbaguidi, G. & Staels, B. Macrophage phenotypes in atherosclerosis. *Immunological reviews* **262**, 153-166 (2014).
114. Sindrilaru, A. & Scharffetter-Kochanek, K. Disclosure of the Culprits: Macrophages-Versatile Regulators of Wound Healing. *Advances in wound care* **2**, 357-368 (2013).
115. Weidenbusch, M. & Anders, H.J. Tissue microenvironments define and get reinforced by macrophage phenotypes in homeostasis or during inflammation, repair and fibrosis. *Journal of innate immunity* **4**, 463-477 (2012).
116. Ruffell, B. & Coussens, L.M. Macrophages and therapeutic resistance in cancer. *Cancer cell* **27**, 462-472 (2015).
117. Harris, R.A. Spatial, Temporal, and Functional Aspects of Macrophages during "The Good, the Bad, and the Ugly" Phases of Inflammation. *Frontiers in immunology* **5**, 612 (2014).
118. Wells, C.A. *et al.* Genetic control of the innate immune response. *BMC immunology* **4**, 5 (2003).
119. Schneemann, M. & Schoeden, G. Macrophage biology and immunology: man is not a mouse. *Journal of leukocyte biology* **81**, 579; discussion 580 (2007).
120. Bogdan, C. Nitric oxide and the immune response. *Nature immunology* **2**, 907-916 (2001).
121. Choi, H.S., Rai, P.R., Chu, H.W., Cool, C. & Chan, E.D. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *American journal of respiratory and critical care medicine* **166**, 178-186 (2002).
122. Cortez-Retamozo, V. *et al.* Origins of tumor-associated macrophages and neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 2491-2496 (2012).
123. Linde, N. *et al.* Vascular endothelial growth factor-induced skin carcinogenesis depends on recruitment and alternative activation of macrophages. *The Journal of pathology* **227**, 17-28 (2012).
124. Noy, R. & Pollard, J.W. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* **41**, 49-61 (2014).

125. Qian, B.Z. *et al.* CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* **475**, 222-225 (2011).

126. Panni, R.Z., Linehan, D.C. & DeNardo, D.G. Targeting tumor-infiltrating macrophages to combat cancer. *Immunotherapy* **5**, 1075-1087 (2013).

127. Bakema, J.E. & van Egmond, M. Fc receptor-dependent mechanisms of monoclonal antibody therapy of cancer. *Current topics in microbiology and immunology* **382**, 373-392 (2014).

128. Urban, J.L. Macrophage-induced enhancement of endogenous tumor lysosome activity. *Cancer research* **41**, 2221-2229 (1981).

129. Martinez, F.O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *Journal of immunology* **177**, 7303-7311 (2006).

130. Biswas, S.K. & Lewis, C.E. NF-kappaB as a central regulator of macrophage function in tumors. *Journal of leukocyte biology* **88**, 877-884 (2010).

131. Hagemann, T., Biswas, S.K., Lawrence, T., Sica, A. & Lewis, C.E. Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. *Blood* **113**, 3139-3146 (2009).

132. Sica, A. *et al.* Macrophage polarization in tumour progression. *Seminars in cancer biology* **18**, 349-355 (2008).

133. Balkwill, F.R. & Mantovani, A. Cancer-related inflammation: common themes and therapeutic opportunities. *Seminars in cancer biology* **22**, 33-40 (2012).

134. Ohri, C.M., Shikotra, A., Green, R.H., Waller, D.A. & Bradding, P. Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. *The European respiratory journal* **33**, 118-126 (2009).

135. Ohri, C.M., Shikotra, A., Green, R.H., Waller, D.A. & Bradding, P. The tissue microlocalisation and cellular expression of CD163, VEGF, HLA-DR, iNOS, and MRP 8/14 is correlated to clinical outcome in NSCLC. *PloS one* **6**, e21874 (2011).

136. Lin, E.Y., Nguyen, A.V., Russell, R.G. & Pollard, J.W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *The Journal of experimental medicine* **193**, 727-740 (2001).

137. Qian, B.Z. & Pollard, J.W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39-51 (2010).

138. Goswami, S. *et al.* Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer research* **65**, 5278-5283 (2005).

139. Wyckoff, J. *et al.* A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer research* **64**, 7022-7029 (2004).

140. Wyckoff, J.B. *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer research* **67**, 2649-2656 (2007).

141. Hernandez, L. *et al.* The EGF/CSF-1 paracrine invasion loop can be triggered by heregulin beta1 and CXCL12. *Cancer research* **69**, 3221-3227 (2009).

142. Muller, A. *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56 (2001).

143. Tsai, M.S., Shamon-Taylor, L.A., Mehmi, I., Tang, C.K. & Lupu, R. Blockage of heregulin expression inhibits tumorigenicity and metastasis of breast cancer. *Oncogene* **22**, 761-768 (2003).

144. Sangaletti, S. *et al.* Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer research* **68**, 9050-9059 (2008).

145. Lu, P., Takai, K., Weaver, V.M. & Werb, Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor perspectives in biology* **3** (2011).

146. Dirkx, A.E., Oude Egbrink, M.G., Wagstaff, J. & Griffioen, A.W. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *Journal of leukocyte biology* **80**, 1183-1196 (2006).

147. De Palma, M. *et al.* Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer cell* **8**, 211-226 (2005).

148. Murdoch, C., Muthana, M., Coffelt, S.B. & Lewis, C.E. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* **8**, 618-631 (2008).

149. Qian, B. *et al.* A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PloS one* **4**, e6562 (2009).

150. Zhang, Q.W. *et al.* Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PloS one* **7**, e50946 (2012).

151. Ong, S.M. *et al.* Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *European journal of immunology* **42**, 89-100 (2012).

152. Edin, S., Wikberg, M.L., Rutegard, J., Oldenborg, P.A. & Palmqvist, R. Phenotypic skewing of macrophages *in vitro* by secreted factors from colorectal cancer cells. *PloS one* **8**, e74982 (2013).

153. Wu, T.H. *et al.* Culture supernatants of different colon cancer cell lines induce specific phenotype switching and functional alteration of THP-1 cells. *Cellular immunology* **290**, 107-115 (2014).

154. Norton, S.E., Ward-Hartstonge, K.A., Taylor, E.S. & Kemp, R.A. Immune cell interplay in colorectal cancer prognosis. *World journal of gastrointestinal oncology* **7**, 221-232 (2015).

155. Forssell, J. *et al.* High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 1472-1479 (2007).

156. Kang, J.C., Chen, J.S., Lee, C.H., Chang, J.J. & Shieh, Y.S. Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. *Journal of surgical oncology* **102**, 242-248 (2010).

157. Kitamura, T. *et al.* SMAD4-deficient intestinal tumors recruit CCR1+ myeloid cells that promote invasion. *Nature genetics* **39**, 467-475 (2007).

158. Cardoso, A.P. *et al.* Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. *Oncogene* **33**, 2123-2133 (2014).

159. Barros, M.H., Hauck, F., Dreyer, J.H., Kempkes, B. & Niedobitek, G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PloS one* **8**, e80908 (2013).

160. Murray, P.J. & Wynn, T.A. Obstacles and opportunities for understanding macrophage polarization. *Journal of leukocyte biology* **89**, 557-563 (2011).

161. Condeelis, J. & Pollard, J.W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266 (2006).

162. De Palma, M. & Lewis, C.E. Macrophage regulation of tumor responses to anticancer therapies. *Cancer cell* **23**, 277-286 (2013).

163. Mantovani, A. & Sica, A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Current opinion in immunology* **22**, 231-237 (2010).

164. Bernhard, E.J. Interventions that induce modifications in the tumor microenvironment. *Cancer radiotherapie : journal de la Societe francaise de radiotherapie oncologique* **15**, 376-382 (2011).

165. Ostuni, R., Kratochvill, F., Murray, P.J. & Natoli, G. Macrophages and cancer: from mechanisms to therapeutic implications. *Trends in immunology* **36**, 229-239 (2015).

166. Li, X. *et al.* Targeting of tumour-infiltrating macrophages via CCL2/CCR2 signalling as a therapeutic strategy against hepatocellular carcinoma. *Gut* (2015).

167. Giraudo, E., Inoue, M. & Hanahan, D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *The Journal of clinical investigation* **114**, 623-633 (2004).

168. Tsagozis, P., Eriksson, F. & Pisa, P. Zoledronic acid modulates antitumoral responses of prostate cancer-tumor associated macrophages. *Cancer immunology, immunotherapy : CII* **57**, 1451-1459 (2008).

169. Zeisberger, S.M. *et al.* Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *British journal of cancer* **95**, 272-281 (2006).

170. Boyle, P. & Levin, B. World Cancer Report. Lyon: International Agency for Research on Cancer (IARC); 2008.

171. Jha, A.K., Prasiko, R., Mod, H., Chaurasia, P.P. & Srivastava, R. Radiotherapy for benign diseases. *JNMA; journal of the Nepal Medical Association* **47**, 151-155 (2008).

172. Montero Luis, A. *et al.* Radiation therapy for the treatment of benign vascular, skeletal and soft tissue diseases. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* **10**, 334-346 (2008).

173. Delaney, G., Jacob, S., Featherstone, C. & Barton, M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer* **104**, 1129-1137 (2005).

174. Baskar, R., Lee, K.A., Yeo, R. & Yeoh, K.W. Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences* **9**, 193-199 (2012).

175. Chao, K.S.C., Perez, C.A. & Brady, L.W. *Radiation Oncology: Management Decisions*, 2nd edn. Lippincott-Raven: Philadelphia, 1999.

176. Joiner, M. & Kogel, A.v.d. *Basic Clinical Radiobiology*, 4th edn. Hodder Arnold: Great Britain, 2009.

177. Baskar, R., Dai, J., Wenlong, N., Yeo, R. & Yeoh, K.W. Biological response of cancer cells to radiation treatment. *Frontiers in molecular biosciences* **1**, 24 (2014).

178.Taylor, B.N. & Thompson, A. The International Systems of Units (SI). In: Department of Commerce - USA, editor. 8th ed. Gaithersburg: National Institute of Standards and Technology; 2008. p. 96.

179. Wrixon, A.D. & Barraclough, I. Radiation, People And The Environment. Austria: International Atomic Energy Agency (IAEA); 2004.

180. Society, A.C. Types of radiation used to treat cancer. 2014 [cited 2016 23 January]Available from: http://www.cancer.org/treatment/treatmentsandsideeffects/treatmenttypes/radiation/radiationtherapyprinciples/radiation-therapy-principles-types-of-radiation

181. Allen, B.J., Bezak, E. & Marcu, L.G. Quo vadis radiotherapy? Technological advances and the rising problems in cancer management. *BioMed research international* **2013**, 749203 (2013).

182. Orecchia, R. *et al.* Electron intraoperative treatment in patients with early-stage breast cancer: data update. *Expert review of anticancer therapy* **6**, 605-611 (2006).

183. Shen, J. *et al.* Hypofractionated electron-beam radiation therapy for keloids: retrospective study of 568 cases with 834 lesions. *Journal of radiation research* **56**, 811-817 (2015).

184. Allen, C., Borak, T.B., Tsujii, H. & Nickoloff, J.A. Heavy charged particle radiobiology: using enhanced biological effectiveness and improved beam focusing to advance cancer therapy. *Mutation research* **711**, 150-157 (2011).

185. Nickoloff, J.A. Photon, light ion, and heavy ion cancer radiotherapy: paths from physics and biology to clinical practice. *Annals of translational medicine* **3**, 336 (2015).

186. Jermann, M. Particle Therapy Statistics in 2014. *Int J Particle Ther* **2**, 50-54 (2015).

187. Barnett, G.C. *et al.* Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. *Nat Rev Cancer* **9**, 134-142 (2009).

188. Withers, H. The four R´s of Radiotherapy. In: Adler, H., Lett, J.T. & Zelle, M. (eds). *Advances in radiation biology*, vol. 5. Academic Press: New York, 1975, pp 241-271.

189. Steel, G.G., McMillan, T.J. & Peacock, J.H. The 5Rs of radiobiology. *International journal of radiation biology* **56**, 1045-1048 (1989).

190. Lidder, P.G. & Hosie, K.B. Rectal cancer: the role of radiotherapy. *Digestive surgery* **22**, 41-48; discussion 49 (2005).

191. Kye, B.H. & Cho, H.M. Overview of radiation therapy for treating rectal cancer. *Annals of coloproctology* **30**, 165-174 (2014).

192. Sermeus, A., Engels, B., Urbain, D. & De Ridder, M. Advances in radiotherapy delivery for rectal cancer: a European perspective. *Expert review of gastroenterology & hepatology* **9**, 393-397 (2015).

193. Bujko, K. *et al.* Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer. *The British journal of surgery* **93**, 1215-1223 (2006).

194. Glimelius, B. Neo-adjuvant radiotherapy in rectal cancer. *World journal of gastroenterology* **19**, 8489-8501 (2013).

195. Seiwert, T.Y., Salama, J.K. & Vokes, E.E. The concurrent chemoradiation paradigm--general principles. *Nature clinical practice. Oncology* **4**, 86-100 (2007).

196. Engelen, S.M. *et al.* Modern multidisciplinary treatment of rectal cancer based on staging with magnetic resonance imaging leads to excellent local control, but distant control remains a challenge. *European journal of cancer* **49**, 2311-2320 (2013).

197. Hoffe, S.E., Shridhar, R. & Biagioli, M.C. Radiation therapy for rectal cancer: current status and future directions. *Cancer control : journal of the Moffitt Cancer Center* **17**, 25-34 (2010).

198. Conde-Muino, R. *et al.* Predictive Biomarkers to Chemoradiation in Locally Advanced Rectal Cancer. *BioMed research international* **2015**, 921435 (2015).

199. Negri, F.V. *et al.* Biological predictive factors in rectal cancer treated with preoperative radiotherapy or radiochemotherapy. *British journal of cancer* **98**, 143-147 (2008).

200. Glimelius, B., Tiret, E., Cervantes, A., Arnold, D. & Group, E.G.W. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **24 Suppl 6**, vi81-88 (2013).

201. Kleiman, L.B., Krebs, A.M., Kim, S.Y., Hong, T.S. & Haigis, K.M. Comparative analysis of radiosensitizers for K-RAS mutant rectal cancers. *PloS one* **8**, e82982 (2013).

202. Shabo, I., Olsson, H., Sun, X.F. & Svanvik, J. Expression of the macrophage antigen CD163 in rectal cancer cells is associated with early local recurrence and reduced survival time. *International journal of cancer. Journal international du cancer* **125**, 1826-1831 (2009).

203. Shaikh, S., Noshirwani, A., West, N., Perry, S. & Jayne, D. Can macrophages within the microenvironment of locally invasive rectal cancers predict response to radiotherapy? Spring Meeting for Clinician Scientists in Training; 2015: The Lancet; 2015. p. S87.

204. Azzam, E.I., Jay-Gerin, J.P. & Pain, D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer letters* **327**, 48-60 (2012).

205. Painter, R.B. DNA damage and repair in eukaryotic cells. *Genetics* **78**, 139-148 (1974).

206. Nambiar, D., Rajamani, P. & Singh, R.P. Effects of phytochemicals on ionization radiation-mediated carcinogenesis and cancer therapy. *Mutation research* **728**, 139-157 (2011).

207. Sulli, G., Di Micco, R. & d'Adda di Fagagna, F. Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. *Nat Rev Cancer* **12**, 709-720 (2012).

208. Bartek, J. & Lukas, J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer cell* **3**, 421-429 (2003).

209. Mah, L.J., El-Osta, A. & Karagiannis, T.C. gammaH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia* **24**, 679-686 (2010).

210. Cervelli, T. *et al.* Effects of single and fractionated low-dose irradiation on vascular endothelial cells. *Atherosclerosis* **235**, 510-518 (2014).

211. Osaki, J.H., Espinha, G., Magalhaes, Y.T. & Forti, F.L. Modulation of RhoA GTPase Activity Sensitizes Human Cervix Carcinoma Cells to gamma-Radiation by Attenuating DNA Repair Pathways. *Oxidative medicine and cellular longevity* **2016**, 6012642 (2016).

212. Santivasi, W.L. & Xia, F. Ionizing radiation-induced DNA damage, response, and repair. *Antioxidants & redox signaling* **21**, 251-259 (2014).

213. Kavanagh, J.N., Redmond, K.M., Schettino, G. & Prise, K.M. DNA double strand break repair: a radiation perspective. *Antioxidants & redox signaling* **18**, 2458-2472 (2013).

214. Cann, K.L. & Hicks, G.G. Regulation of the cellular DNA double-strand break response. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **85**, 663-674 (2007).

215. Vakifahmetoglu, H., Olsson, M. & Zhivotovsky, B. Death through a tragedy: mitotic catastrophe. *Cell death and differentiation* **15**, 1153-1162 (2008).

216. Bucci, B. *et al.* Fractionated ionizing radiation exposure induces apoptosis through caspase-3 activation and reactive oxygen species generation. *Anticancer research* **26**, 4549-4557 (2006).

217. Bernier, J., Hall, E.J. & Giaccia, A. Radiation oncology: a century of achievements. *Nat Rev Cancer* **4**, 737-747 (2004).

218. Begg, A.C., Stewart, F.A. & Vens, C. Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* **11**, 239-253 (2011).

219. Dillon, M.T., Good, J.S. & Harrington, K.J. Selective targeting of the G2/M cell cycle checkpoint to improve the therapeutic index of radiotherapy. *Clinical oncology* **26**, 257-265 (2014).

220. Matsumoto, Y. *et al.* Chapter 23 - Radiosensitization Strategies Through Modification of DNA Double-Strand Break Repair. In: Chen, C., editor. *New Research Directions in DNA Repair*: InTech; 2013. pp. 639-662.

221. Kam, W.W. & Banati, R.B. Effects of ionizing radiation on mitochondria. *Free radical biology & medicine* **65**, 607-619 (2013).

222. Haimovitz-Friedman, A. *et al.* Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *The Journal of experimental medicine* **180**, 525-535 (1994).

223. Kolesnick, R. & Fuks, Z. Radiation and ceramide-induced apoptosis. *Oncogene* **22**, 5897-5906 (2003).

224. Heinrich, M. *et al.* Cathepsin D targeted by acid sphingomyelinase-derived ceramide. *The EMBO journal* **18**, 5252-5263 (1999).

225. Heinrich, M. *et al.* Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell death and differentiation* **11**, 550-563 (2004).

226. Corre, I., Guillonneau, M. & Paris, F. Membrane signaling induced by high doses of ionizing radiation in the endothelial compartment. Relevance in radiation toxicity. *International journal of molecular sciences* **14**, 22678-22696 (2013).

227. Corre, I., Niaudet, C. & Paris, F. Plasma membrane signaling induced by ionizing radiation. *Mutation research* **704**, 61-67 (2010).

228. Stancevic, B. & Kolesnick, R. Ceramide-rich platforms in transmembrane signaling. *FEBS letters* **584**, 1728-1740 (2010).

229. Bionda, C. *et al.* Radioresistance of human carcinoma cells is correlated to a defect in raft membrane clustering. *Free radical biology & medicine* **43**, 681-694 (2007).

230. Lemmon, M.A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134 (2010).

231. Valerie, K. *et al.* Radiation-induced cell signaling: inside-out and outside-in. *Molecular cancer therapeutics* **6**, 789-801 (2007).

232. Yacoub, A. *et al.* Radiotherapy-induced signal transduction. *Endocrine-related cancer* **13 Suppl 1**, S99-114 (2006).

233. Munshi, A. & Ramesh, R. Mitogen-activated protein kinases and their role in radiation response. *Genes & cancer* **4**, 401-408 (2013).

234. Hein, A.L., Ouellette, M.M. & Yan, Y. Radiation-induced signaling pathways that promote cancer cell survival (review). *International journal of oncology* **45**, 1813-1819 (2014).

235. De Bacco, F. *et al.* Induction of MET by ionizing radiation and its role in radioresistance and invasive growth of cancer. *Journal of the National Cancer Institute* **103**, 645-661 (2011).

236. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annual review of immunology* **27**, 693-733 (2009).

237.Perkins, N.D. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nature reviews. Molecular cell biology* **8**, 49-62 (2007).

238. Gerondakis, S., Fulford, T.S., Messina, N.L. & Grumont, R.J. NF-kappaB control of T cell development. *Nature immunology* **15**, 15-25 (2014).

239. Shih, V.F., Tsui, R., Caldwell, A. & Hoffmann, A. A single NFkappaB system for both canonical and non-canonical signaling. *Cell research* **21**, 86-102 (2011).

240. Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**, 6853-6866 (1999).

241. Brzoska, K. & Szumiel, I. Signalling loops and linear pathways: NF-kappaB activation in response to genotoxic stress. *Mutagenesis* **24**, 1-8 (2009).

242. Habraken, Y. & Piette, J. NF-kappaB activation by double-strand breaks. *Biochemical pharmacology* **72**, 1132-1141 (2006).

243. Ahmed, K.M. & Li, J.J. NF-kappa B-mediated adaptive resistance to ionizing radiation. *Free radical biology & medicine* **44**, 1-13 (2008).

244. Liu, Y.C., Chiang, I.T., Hsu, F.T. & Hwang, J.J. Using NF-kappaB as a molecular target for theranostics in radiation oncology research. *Expert review of molecular diagnostics* **12**, 139-146 (2012).

245. Hellweg, C.E. The Nuclear Factor kappaB pathway: A link to the immune system in the radiation response. *Cancer letters* **368**, 275-289 (2015).

246. Lin, R.X. *et al.* Proteomic analysis of ionizing radiation-induced proteins at the subcellular level. *Journal of proteome research* **8**, 390-399 (2009).

247. Bhatt, A.N. *et al.* Transient elevation of glycolysis confers radio-resistance by facilitating DNA repair in cells. *BMC cancer* **15**, 335 (2015).

248. Lynam-Lennon, N. *et al.* Altered mitochondrial function and energy metabolism is associated with a radioresistant phenotype in oesophageal adenocarcinoma. *PloS one* **9**, e100738 (2014).

249. Bing, Z. *et al.* Proteomic analysis of effects by x-rays and heavy ion in HeLa cells. *Radiology and oncology* **48**, 142-154 (2014).

250. Barker, H.E., Paget, J.T., Khan, A.A. & Harrington, K.J. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer* **15**, 409-425 (2015).

251. Joye, I. & Haustermans, K. Early and late toxicity of radiotherapy for rectal cancer. *Recent results in cancer research. Fortschritte der Krebsforschung. Progres dans les recherches sur le cancer* **203**, 189-201 (2014).

252. Mao, X.W. A quantitative study of the effects of ionizing radiation on endothelial cells and capillary-like network formation. *Technology in cancer research & treatment* **5**, 127-134 (2006).

253. Langley, R.E., Bump, E.A., Quartuccio, S.G., Medeiros, D. & Braunhut, S.J. Radiation-induced apoptosis in microvascular endothelial cells. *British journal of cancer* **75**, 666-672 (1997).

254. de Oliveira, I. The effects of low-dose ionizing radiation on angiogenesis. PhD in Biology thesis, University of Lisbon - Faculty of Sciences, Lisbon, 2011.

255. Sofia Vala, I. *et al.* Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. *PloS one* **5**, e11222 (2010).

256. Chang, C.C. *et al.* Dose-dependent effect of radiation on angiogenic and angiostatic CXC chemokine expression in human endothelial cells. *Cytokine* **48**, 295-302 (2009).

257. Meeren, A.V., Bertho, J.M., Vandamme, M. & Gaugler, M.H. Ionizing radiation enhances IL-6 and IL-8 production by human endothelial cells. *Mediators of inflammation* **6**, 185-193 (1997).

258. Igarashi, K., Sakimoto, I., Kataoka, K., Ohta, K. & Miura, M. Radiation-induced senescence-like phenotype in proliferating and plateau-phase vascular endothelial cells. *Experimental cell research* **313**, 3326-3336 (2007).

259. Paris, F. *et al.* Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* **293**, 293-297 (2001).

260. Wang, J., Boerma, M., Fu, Q. & Hauer-Jensen, M. Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy. *World journal of gastroenterology* **13**, 3047-3055 (2007).

261. Fajardo, L.F. The pathology of ionizing radiation as defined by morphologic patterns. *Acta oncologica* **44**, 13-22 (2005).

262. Russell, N.S. *et al.* Novel insights into pathological changes in muscular arteries of radiotherapy patients. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* **92**, 477-483 (2009).

263. Ding, N.H., Li, J.J. & Sun, L.Q. Molecular mechanisms and treatment of radiation-induced lung fibrosis. *Current drug targets* **14**, 1347-1356 (2013).

264. Shaw, T.J. & Martin, P. Wound repair at a glance. *Journal of cell science* **122**, 3209-3213 (2009).

265. Heylmann, D., Rodel, F., Kindler, T. & Kaina, B. Radiation sensitivity of human and murine peripheral blood lymphocytes, stem and progenitor cells. *Biochimica et biophysica acta* **1846**, 121-129 (2014).

266. Kim, J.H., Jenrow, K.A. & Brown, S.L. Mechanisms of radiation-induced normal tissue toxicity and implications for future clinical trials. *Radiation oncology journal* **32**, 103-115 (2014).

267. Gough, M.J., Young, K. & Crittenden, M. The impact of the myeloid response to radiation therapy. *Clinical & developmental immunology* **2013**, 281958 (2013).

268. Donaldson, D.M., Marcus, S., Gyi, K.K. & Perkins, E.H. The influence of immunization and total body x-irradiation on intracellular digestion by peritoneal phagocytes. *Journal of immunology* **76**, 192-199 (1956).

269. Geiger, B. & Gallily, R. Effect of X-irradiation on various functions of murine macrophages. *Clinical and experimental immunology* **16**, 643-655 (1974).

270. Schmidtke, J.R. & Dixon, F.J. The functional capacity of x-irradiated macrophages. *Journal of immunology* **108**, 1624-1630 (1972).

271. Sablonniere, B., Nicolas, J., Neveux, Y. & Drouet, J. Effect of whole-body irradiation on phagocytic activity of rat alveolar macrophages. *International journal of radiation biology and related studies in physics, chemistry, and medicine* **44**, 575-584 (1983).

272. Gallin, E.K. & Green, S.W. Exposure to gamma-irradiation increases phorbol myristate acetate-induced H2O2 production in human macrophages. *Blood* **70**, 694-701 (1987).

273. Gallin, E.K., Green, S.W. & Sheehy, P.A. Enhanced activity of the macrophage-like cell line J774.1 following exposure to gamma radiation. *Journal of leukocyte biology* **38**, 369-381 (1985).

274. Lambert, L.E. & Paulnock, D.M. Modulation of macrophage function by gamma-irradiation. Acquisition of the primed cell intermediate stage of the macrophage tumoricidal activation pathway. *Journal of immunology* **139**, 2834-2841 (1987).

275. Duerst, R. & Werberig, K. Cells of the J774 macrophage cell line are primed for antibody-dependent cell-mediated cytotoxicity following exposure to gamma-irradiation. *Cellular immunology* **136**, 361-372 (1991).

276. McLennan, G., Oberley, L.W. & Autor, A.P. The role of oxygen-derived free radicals in radiation-induced damage and death of nondividing eucaryotic cells. *Radiation research* **84**, 122-132 (1980).

277. McKinney, L.C., Aquilla, E.M., Coffin, D., Wink, D.A. & Vodovotz, Y. Ionizing radiation potentiates the induction of nitric oxide synthase by IFN-gamma and/or LPS in murine macrophage cell lines: role of TNF-alpha. *Journal of leukocyte biology* **64**, 459-466 (1998).

278. Nowosielska, E.M., Wrembel-Wargocka, J., Cheda, A., Lisiak, E. & Janiak, M.K. Enhanced cytotoxic activity of macrophages and suppressed tumor metastases in mice irradiated with low doses of X- rays. *Journal of radiation research* **47**, 229-236 (2006).

279. Ibuki, Y., Mizuno, S. & Goto, R. gamma-Irradiation-induced DNA damage enhances NO production via NF-kappaB activation in RAW264.7 cells. *Biochimica et biophysica acta* **1593**, 159-167 (2003).

280. Shan, Y.X., Jin, S.Z., Liu, X.D., Liu, Y. & Liu, S.Z. Ionizing radiation stimulates secretion of pro-inflammatory cytokines: dose-response relationship, mechanisms and implications. *Radiation and environmental biophysics* **46**, 21-29 (2007).

281. Chen, H. *et al.* Reactive oxygen species and x-ray disrupted spontaneous [Ca(2)(+)]I oscillation in alveolar macrophages. *Radiation research* **179**, 485-492 (2013).

282. Olwell, P.M. *et al.* Cytoskeletal reorganization and altered phagocytotic ability in primary cultures of rainbow trout hemopoietic tissue exposed to low-level ionizing radiation. *Radiation research* **164**, 45-52 (2005).

283. Conrad, S., Ritter, S., Fournier, C. & Nixdorff, K. Differential effects of irradiation with carbon ions and x-rays on macrophage function. *Journal of radiation research* **50**, 223-231 (2009).

284. Benacerraf, B. Influence of irradiation on resistance to infection. *Bacteriological reviews* **24**, 35-40 (1960).

285. Benacerraf, B., Kivy-Rosenberg, E., Sebestyen, M.M. & Zweifach, B.W. The effect of high doses of x-irradiation on the phagocytic, proliferative, and metabolic properties of the reticulo-endothelial system. *The Journal of experimental medicine* **110**, 49-64 (1959).

286. Chen, C., Boylan, M.T., Evans, C.A., Whetton, A.D. & Wright, E.G. Application of two-dimensional difference gel electrophoresis to studying bone marrow macrophages and their *in vivo* responses to ionizing radiation. *Journal of proteome research* **4**, 1371-1380 (2005).

287. Kubota, Y., Kinoshita, K., Suetomi, K., Fujimori, A. & Takahashi, S. Mcl-1 depletion in apoptosis elicited by ionizing radiation in peritoneal resident macrophages of C3H mice. *Journal of immunology* **178**, 2923-2931 (2007).

288. Smallwood, H.S., Lopez-Ferrer, D., Eberlein, P.E., Watson, D.J. & Squier, T.C. Calmodulin mediates DNA repair pathways involving H2AX in response to low-dose radiation exposure of RAW 264.7 macrophages. *Chemical research in toxicology* **22**, 460-470 (2009).

289. Bauer, M. *et al.* Human monocytes are severely impaired in base and DNA double-strand break repair that renders them vulnerable to oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 21105-21110 (2011).

290. Pavlopouloua, A. *et al.* Unraveling the mechanisms of extreme radioresistance in prokaryotes: Lessons from nature. *In press*. *Mutat Res/Rev in Mutat Res* (2015).

291. Liu, S.Z., Jin, S.Z., Liu, X.D. & Sun, Y.M. Role of CD28/B7 costimulation and IL-12/IL-10 interaction in the radiation-induced immune changes. *BMC immunology* **2**, 8 (2001).

292. Pandey, R., Shankar, B.S., Sharma, D. & Sainis, K.B. Low dose radiation induced immunomodulation: effect on macrophages and CD8+ T cells. *International journal of radiation biology* **81**, 801-812 (2005).

293. Tasat, D.R. *et al.* Radiation effects on oxidative metabolism in young and aged rat alveolar macrophages. *Cellular and molecular biology* **48**, 529-535 (2002).

294. Zhou, Y. *et al.* Modulation of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in RAW264.7 cells by irradiation. *Molecular medicine reports* **3**, 809-813 (2010).

295. Frischholz, B. *et al.* Reduced secretion of the inflammatory cytokine IL-1beta by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation. *Autoimmunity* **46**, 323-328 (2013).

296. Wunderlich, R. *et al.* Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. *Clinical and experimental immunology* **179**, 50-61 (2015).

297. Lodermann, B. *et al.* Low dose ionising radiation leads to a NF-kappaB dependent decreased secretion of active IL-1beta by activated macrophages with a discontinuous dose-dependency. *International journal of radiation biology* **88**, 727-734 (2012).

298. Tsukimoto, M., Homma, T., Mutou, Y. & Kojima, S. 0.5 Gy gamma radiation suppresses production of TNF-alpha through up-regulation of MKP-1 in mouse macrophage RAW264.7 cells. *Radiation research* **171**, 219-224 (2009).

299. Katayama, I., Hotokezaka, Y., Matsuyama, T., Sumi, T. & Nakamura, T. Ionizing radiation induces macrophage foam cell formation and aggregation through JNK-dependent activation of CD36 scavenger receptors. *International journal of radiation oncology, biology, physics* **70**, 835-846 (2008).

300. Quast, U. Whole body radiotherapy: A TBI-guideline. *Journal of medical physics / Association of Medical Physicists of India* **31**, 5-12 (2006).

301. Kuten, A., Rosenblatt, E., Dale, J., Leviov, M. & Tatcher, M. Total skin electron irradiation: efficacy in early mycosis fungoides. *Leukemia & lymphoma* **10**, 281-285 (1993).

302. Stewart, F.A. *et al.* Ionizing radiation accelerates the development of atherosclerotic lesions in ApoE-/- mice and predisposes to an inflammatory plaque phenotype prone to hemorrhage. *The American journal of pathology* **168**, 649-658 (2006).

303. Williams, J.P., Johnston, C.J. & Finkelstein, J.N. Treatment for radiation-induced pulmonary late effects: spoiled for choice or looking in the wrong direction? *Current drug targets* **11**, 1386-1394 (2010).

304. Abbott, A. Researchers pin down risks of low-dose radiation. *Nature* **523**, 17-18 (2015).

305. Mullenders, L., Atkinson, M., Paretzke, H., Sabatier, L. & Bouffler, S. Assessing cancer risks of low-dose radiation. *Nat Rev Cancer* **9**, 596-604 (2009).

306. UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation). Biological mechanisms of radiation actions at low doses. A white paper to guide the Scientific Committee's future programme of work. New York: United Nations; 2012.

307. Rodel, F. *et al.* Immunomodulatory properties and molecular effects in inflammatory diseases of low-dose x-irradiation. *Frontiers in oncology* **2**, 120 (2012).

308. Chiang, C.S. *et al.* Irradiation promotes an m2 macrophage phenotype in tumor hypoxia. *Frontiers in oncology* **2**, 89 (2012).

309. Garcia-Barros, M. *et al.* Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* **300**, 1155-1159 (2003).

310. Hobson, B. & Denekamp, J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *British journal of cancer* **49**, 405-413 (1984).

311. Abdollahi, A. *et al.* Inhibition of alpha(v)beta3 integrin survival signaling enhances antiangiogenic and antitumor effects of radiotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 6270-6279 (2005).

312. Li, M., Jendrossek, V. & Belka, C. The role of PDGF in radiation oncology. *Radiation oncology* **2**, 5 (2007).

313. Abdollahi, A. *et al.* SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation *in vitro*. *Cancer research* **63**, 3755-3763 (2003).

314. Gray, L.H., Conger, A.D., Ebert, M., Hornsey, S. & Scott, O.C. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British journal of radiology* **26**, 638-648 (1953).

315. Dewhirst, M.W. Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. *Radiation research* **172**, 653-665 (2009).

316. Fenton, B.M., Lord, E.M. & Paoni, S.F. Effects of radiation on tumor intravascular oxygenation, vascular configuration, development of hypoxia, and clonogenic survival. *Radiation research* **155**, 360-368 (2001).

317. Harada, H. *et al.* The Akt/mTOR pathway assures the synthesis of HIF-1alpha protein in a glucose- and reoxygenation-dependent manner in irradiated tumors. *The Journal of biological chemistry* **284**, 5332-5342 (2009).

318. Martinive, P. *et al.* Preconditioning of the tumor vasculature and tumor cells by intermittent hypoxia: implications for anticancer therapies. *Cancer research* **66**, 11736-11744 (2006).

319. Rofstad, E.K., Galappathi, K., Mathiesen, B. & Ruud, E.B. Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 1971-1978 (2007).

320. Chen, F.H. *et al.* Radiotherapy decreases vascular density and causes hypoxia with macrophage aggregation in TRAMP-C1 prostate tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 1721-1729 (2009).

321. Brown, J.M. Vasculogenesis: a crucial player in the resistance of solid tumours to radiotherapy. *The British journal of radiology* **87**, 20130686 (2014).

322. Galluzzi, L., Kepp, O. & Kroemer, G. Immunogenic cell death in radiation therapy. *Oncoimmunology* **2**, e26536 (2013).

323. Kroemer, G., Galluzzi, L., Kepp, O. & Zitvogel, L. Immunogenic cell death in cancer therapy. *Annual review of immunology* **31**, 51-72 (2013).

324. Krysko, D.V. *et al.* Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* **12**, 860-875 (2012).

325. Golden, E.B., Pellicciotta, I., Demaria, S., Barcellos-Hoff, M.H. & Formenti, S.C. The convergence of radiation and immunogenic cell death signaling pathways. *Frontiers in oncology* **2**, 88 (2012).

326.Vatner, R.E. & Formenti, S.C. Myeloid-derived cells in tumors: effects of radiation. *Seminars in radiation oncology* **25**, 18-27 (2015).

327.Persa, E., Balogh, A., Safrany, G. & Lumniczky, K. The effect of ionizing radiation on regulatory T cells in health and disease. *Cancer letters* **368**, 252-261 (2015).

328.Huo, L., Nagasawa, H. & Little, J.B. HPRT mutants induced in bystander cells by very low fluences of alpha particles result primarily from point mutations. *Radiation research* **156**, 521-525 (2001).

329.Mothersill, C. & Seymour, C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International journal of radiation biology* **71**, 421-427 (1997).

330.Sawant, S.G., Randers-Pehrson, G., Geard, C.R., Brenner, D.J. & Hall, E.J. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells *in vitro* can be initiated in the unirradiated neighbors of irradiated cells. *Radiation research* **155**, 397-401 (2001).

331.Yang, H., Asaad, N. & Held, K.D. Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. *Oncogene* **24**, 2096-2103 (2005).

332.Morgan, W.F. & Sowa, M.B. Effects of ionizing radiation in nonirradiated cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14127-14128 (2005).

333. Nagasawa, H. & Little, J.B. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer research* **52**, 6394-6396 (1992).

334. Gerashchenko, B.I. & Howell, R.W. Cell proximity is a prerequisite for the proliferative response of bystander cells co-cultured with cells irradiated with gamma-rays. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **56**, 71-80 (2003).

335. Belyakov, O.V. *et al.* Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14203-14208 (2005).

336. Morgan, W.F. & Sowa, M.B. Non-targeted bystander effects induced by ionizing radiation. *Mutation research* **616**, 159-164 (2007).

337. Belyakov, O.V., Folkard, M., Mothersill, C., Prise, K.M. & Michael, B.D. Bystander-induced differentiation: a major response to targeted irradiation of a urothelial explant model. *Mutation research* **597**, 43-49 (2006).

338. yer, R. & Lehnert, B.E. Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. *Mutation research* **503**, 1-9 (2002).

339. Lyng, F.M., Seymour, C.B. & Mothersill, C. Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for bystander-induced genomic instability? *Radiation research* **157**, 365-370 (2002).

340. Morgan, W.F. & Sowa, M.B. Non-targeted effects induced by ionizing radiation: mechanisms and potential impact on radiation induced health effects. *Cancer letters* **356**, 17-21 (2015).

341. Prise, K.M. & O'Sullivan, J.M. Radiation-induced bystander signalling in cancer therapy. *Nat Rev Cancer* **9**, 351-360 (2009).

342. Formenti, S.C. & Demaria, S. Systemic effects of local radiotherapy. *The Lancet. Oncology* **10**, 718-726 (2009).

343. Dong, C. *et al.* The differential role of human macrophage in triggering secondary bystander effects after either gamma-ray or carbon beam irradiation. *Cancer letters* **363**, 92-100 (2015).

344. Lodge, M. *et al.* A systematic literature review of the clinical and cost-effectiveness of hadron therapy in cancer. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* **83**, 110-122 (2007).

345. Lu, B., Kim, D.W. & Hallahan, D.E. Tumor angiogenesis as a strategy for radiosensitization. *Cancer Therapy* **1**, 335-342 (2003).

346. Martin, B.J. Inhibiting vasculogenesis after radiation: a new paradigm to improve local control by radiotherapy. *Seminars in radiation oncology* **23**, 281-287 (2013).

347. Galluzzi, L. *et al.* Classification of current anticancer immunotherapies. *Oncotarget* **5**, 12472-12508 (2014).

348. Kyi, C. & Postow, M.A. Checkpoint blocking antibodies in cancer immunotherapy. *FEBS letters* **588**, 368-376 (2014).

349. Cancer Research Institute. Timeline of Progress. [cited 2016 4 Jan]Available from: http://www.cancerresearch.org/our-strategy-impact/timeline-of-progress/timeline-detail

350. Formenti, S.C. & Demaria, S. Combining radiotherapy and cancer immunotherapy: a paradigm shift. *Journal of the National Cancer Institute* **105**, 256-265 (2013).

351. Mantovani, A. & Allavena, P. The interaction of anticancer therapies with tumor-associated macrophages. *The Journal of experimental medicine* **212**, 435-445 (2015).

352. Meng, Y. *et al.* Blockade of tumor necrosis factor alpha signaling in tumor-associated macrophages as a radiosensitizing strategy. *Cancer research* **70**, 1534-1543 (2010).

353. Liu, S.C. *et al.* Blockade of SDF-1 after irradiation inhibits tumor recurrences of autochthonous brain tumors in rats. *Neuro-oncology* **16**, 21-28 (2014).

354. Stafford, J.H. *et al.* Colony stimulating factor 1 receptor inhibition delays recurrence of glioblastoma after radiation by altering myeloid cell recruitment and polarization. *Neuro-oncology* (2015).

355. Xu, J. *et al.* CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer research* **73**, 2782-2794 (2013).

356. Farooque, A., Afrin, F., Adhikari, J.S. & Dwarakanath, B.S. Polarization of macrophages towards M1 phenotype by a combination of 2-deoxy-d-glucose and radiation: Implications for tumor therapy. *Immunobiology* **221**, 269-281 (2016).

357. Cardoso, A.P. *et al.* An interferon-gamma-delivery system based on chitosan/poly(gamma-glutamic acid) polyelectrolyte complexes modulates macrophage-derived stimulation of cancer cell invasion *in vitro*. *Acta biomaterialia* **23**, 157-171 (2015).

358. Fridlender, Z.G. *et al.* Using macrophage activation to augment immunotherapy of established tumours. *British journal of cancer* **108**, 1288-1297 (2013).

359. D'Incalci, M., Badri, N., Galmarini, C.M. & Allavena, P. Trabectedin, a drug acting on both cancer cells and the tumour microenvironment. *British journal of cancer* **111**, 646-650 (2014).

Chapter II

Aims

Despite technological advances in radiotherapy, resistance to treatment is still a major challenge in cancer management, namely in rectal cancer. Tumour-associated host cells, particularly macrophages, contribute to cancer cell migration, invasion, angiogenesis and metastasis, modulating cancer progression. Like many other components of the tumour microenvironment, macrophages are also irradiated when the tumour is subjected to radiotherapy and their response may contribute to treatment outcome. Therefore, it has become fundamental to further explore the activity of macrophages under an irradiation context.

Although a good amount of knowledge about macrophage response to radiation has been generated over the past years, there is scarce information on the role of ionizing radiation on human, not mouse, macrophages and using clinically relevant fractionated schemes rather than single doses. This lacuna in the literature, identified by us in 2010 and still currently observed, was the scientific motivation to design the present PhD thesis. Thus, we aimed to provide new insights into how ionizing radiation, particularly clinically relevant doses, affects human macrophages, and modulates their communication with cancer cells. In order to address these issues, human monocyte-derived macrophages and two colorectal cancer cells lines, RKO and SW1463, were exposed to cumulative ionizing radiation doses (2 Gy/fraction/day), during 5 days, mimicking one week of the neoadjuvant treatment of a colorectal cancer patient. These working models were crucial to address our mains aims, which are summarized as follows:

1. **To understand the effects of ionizing radiation exposure on human macrophages,**

    A. from a functional point a view

    To address this aim, macrophages were irradiated with 2, 6 or 10 Gy cumulative ionizing radiation doses and macrophage DNA damage, pro-survival activity and NF-κB signalling pathway activation were first evaluated. Then, a plethora of macrophage functions like plasticity, proteolysis, phagocytosis and macrophage ability to promote cancer cell invasion and cancer cell-induced angiogenesis was characterized using macrophages irradiated with 5 cumulative ionizing radiation fractions (5 x 2 Gy). This data is presented in *Chapter III* (Research Article 1).

    B. from a signalling perspective

    In order to obtain an overview of the main signalling pathways being affected by ionizing radiation exposure on macrophages, a detailed proteomic study combining gel-based (2-DE) and gel-free (iTRAQ followed by 2D-LC) methodological approaches was performed

It allowed the evaluation of protein expression alterations in irradiated (5 x 2 Gy) macrophages when compared with non-irradiated ones. The deregulated expression of two main targets was validated by western blot analysis and functional studies were also provided. These results are included in *Chapter IV* (Research Article 2).

2. To reveal how ionizing radiation exposure affects macrophage-cancer cell communication

To address this issue, an indirect macrophage-cancer cell co-culture model was established, mimicking more closely the communication of macrophages with cancer cells at the tumour microenvironment. RKO and SW1463 colorectal cancer cells, which exhibit high and low radiosensitivity, respectively, were used as models. The macrophage-cancer cell set was irradiated as a whole for 5 days (5 x 2 Gy) and apoptosis of cancer cells was first addressed, followed by determination of expression alterations in some of their survival and metabolism-related genes. In parallel, the role of cancer cells on macrophage response to radiation was also evaluated through characterization of their capacity to modulate macrophage polarization status. Finally, the ability of irradiated co-cultures to promote the radiation-induced bystander effect was evaluated by exposing non-irradiated cancer cells to conditioned medium from irradiated or non-irradiated co-cultures. These results are included in *Chapter V* (Research Article 3).

The discovery of new molecular targets and the knowledge acquired along this PhD work are expected to contribute for a better understanding of macrophage and cancer cell response to clinically relevant ionizing radiation doses. Ultimately, we believe that these results will be important for the design of further therapeutic strategies aiming to improve radiotherapy efficacy through targeting of the tumour microenvironment.

Chapter III

Research Article 1

Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities

| Sci Rep. 2016 Jan 6; 6:18765. doi: 10.1038/srep18765.

Chapter overview

This chapter contains experimental results addressing the first aim (topic 1A) of the present PhD thesis - *To understand the effects of ionizing radiation exposure on human macrophages, from a functional point a view*. Macrophages irradiated with 2, 6 or 10 Gy cumulative ionizing radiation doses were used as working models. A detailed characterization of macrophage viability and survival (through evaluation of DNA damage, apoptosis and pro-survival signalling, including NF-κB pathway) as well as macrophage functionality (inflammatory profile, plasticity, phagocytosis, MMP-2 and-9 activity, promotion of cancer cell invasion and cancer cell-induced angiogenesis) upon irradiation was performed.

Data is mainly compiled in an original research manuscript untitled "*Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities*", with the respective supplementary information, published at *Scientific Reports*. At the end of this chapter, a brief report of complementary, but not yet published results, is also presented. It aims to explore: the i) NF-κB signalling in irradiated macrophages beyond results described the manuscript as well as ii) the effect of a single 2 Gy dose on macrophages, as this topic was the starting point for the development of the experimental work presented in this chapter.

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Research Article 1

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Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities

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In order to improve the efficacy of conventional radiotherapy, attention has been paid to immune cells, which not only modulate cancer cell response to therapy but are also highly recruited to tumours after irradiation. Particularly, the effect of ionizing radiation on macrophages, using therapeutically relevant doses, is not well understood. To evaluate how radiotherapy affects macrophage behaviour and macrophage-mediated cancer cell activity, human monocyte derived-macrophages were subjected, for a week, to cumulative ionizing radiation doses, as used during cancer treatment (2 Gy/fraction/day). Irradiated macrophages remained viable and metabolically active, despite DNA damage. NF-kappaB transcription activation and increased Bcl-xL expression evidenced the promotion of pro-survival activity. A significant increase of pro-inflammatory macrophage markers *CD80*, CD86 and HLA-DR, but not *CCR7*, *TNF* and *IL1B* was observed after 10 Gy cumulative doses, while anti-inflammatory markers *CD163*, *MRC1*, *VCAN* and IL-10 expression decreased, suggesting the modulation towards a more pro-inflammatory phenotype. Moreover, ionizing radiation induced macrophage morphological alterations and increased their phagocytic rate, without affecting matrix metalloproteases (MMP)2 and MMP9 activity. Importantly, irradiated macrophages promoted cancer cell-invasion and cancer cell-induced angiogenesis. Our work highlights macrophage ability to sustain cancer cell activities as a major concern that needs to be addressed to improve radiotherapy efficacy.

Radiation therapy is a widely used and highly cost effective cancer treatment modality[1,2]. The biological principle of its application relies mainly on the direct effect on cancer cells, as they usually exhibit higher proliferative rates and impairment in DNA repair mechanisms, when compared to host cells[3]. Although physics and technological evolution in the field of radiotherapy, involving new imaging modalities and more advanced software/equipment, have largely contributed to improve local control, it is necessary to improve radiotherapy targeting, control disease progression and predict treatment outcome[4,5]. To achieve the desired effectiveness, new therapeutic strategies,

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administered concomitantly, before or after radiotherapy, are required[6]. The key may rely on a better understanding of the effect of ionizing radiation on tumour stromal cells, as they are crucial for disease progression and treatment outcome, and are also irradiated[7–10]. Ionizing radiation-induced cancer cell death releases death-signals, which lead to the recruitment of more immune cells, including monocytes which differentiate into macrophages at the injured region[11]. Within the different immune cells present at the tumour microenvironment, macrophages are particularly relevant, as they constitute, in many tumours, the major inflammatory stromal component and are also known as obligate partners for cancer cell migration, invasion and metastasis[12,13]. Due to their sophisticated phagocytic ability, macrophages perform a crucial role in clearing dying cells, contributing to the induction of tolerance, or stimulation of adaptive antitumour immunity[11,14].

Nevertheless, it is not well understood how ionizing radiation, namely clinically relevant doses, directly affects macrophage behaviour as well as macrophage regulation of cancer progression. Till now a clear limitation on determining the clinical effect of ionizing radiation on elements of the tumour microenvironment are the model systems analysed and the dose of radiation used. Ionizing radiation for cancer treatment is usually delivered in a multi-fractionated regimen, with daily doses of typically 2 Gy (5×/week), often delivered during several weeks[15]. However, the majority of the studies aiming to reveal the effect of ionizing radiation on macrophages are performed in mouse models and make use of single, low (<0.1 Gy) or moderate (0.1 Gy–1 Gy) doses[16–20], thus not relevant in a human clinical context.

The present work aims to reveal the effect of fractionated ionizing radiation on human macrophages, mimicking a week of cancer patients' treatment. In order to achieve this, human monocyte-derived macrophages were differentiated in the presence of M-CSF, a factor involved in the recruitment of monocytes to the tissues, and exposed to cumulative ionizing radiation doses of 2 Gy each, up to 10 Gy. A plethora of functions in macrophages was then characterized. We demonstrate that irradiated macrophages are viable and metabolically active, activate NF-κB, exhibit a reduced anti-inflammatory profile, increased phagocytosis and unaltered MMP-2 and 9-mediated proteolysis. We also evidenced that irradiated macrophages are still able to promote tumour cell invasion and angiogenesis. Overall, our work adds novelty to the current literature and reinforces the idea of targeting macrophage differentiation and/or their molecular targets as a complementary strategy to improve radiotherapy efficacy.

Results

Irradiated macrophages are viable and metabolically active, despite DNA damage. To confirm DNA damage induced by the fractionated irradiation protocol, 10 Gy irradiated macrophages were fixed and immunostained for phosphorylated H2AX (Ser139) (γH2AX), a sensitive marker of DNA double-strand breaks (DSBs)[21]. Immunofluorescence images and respective quantification indicated that a significantly ($P < 0.01$) higher percentage (87.17 ± 7.99%) of irradiated macrophages presented γH2AX foci, in comparison to non-irradiated ones (6.85 ± 1.03%) (Fig. 1A). Upon ionizing radiation exposure, DNA damage signals are generally sensed and propagated through a kinase cascade, including Ataxia Telangiectasia Mutated Kinase (ATM) and also Checkpoint kinase 2 (Chk2)[22]. Therefore, Chk2 expression and phosphorylation status were evaluated by western blot analysis. Chk2 activation was consistently observed in all donors at 1, 6 and 24 h after cumulative ionizing radiation doses (2, 6 and 10 Gy) (Fig. 1B). This data confirms that the irradiation protocol applied induced macrophage DNA damage, from single to several cumulative doses, and that the triggered signalling response involves Chk2 activation.

Depending on the level of radiation-induced DNA damage, cell death may or not occur[22]. Apoptosis is known as the major cell death modality induced by ionizing radiation in cells from the myeloid lineage, which is composed by progenitor and mature effector myeloid cells, such as macrophages, dendritic cells, erythrocytes and platelets[23–25]. In the last years, a heterogeneous population of immature myeloid cells with immunosuppressive properties, termed myeloid-derived suppressor cells (MDSCs), has been defined[26]. Together with macrophages, this population is frequently recruited into tumours after ionizing radiation exposure and less radiosensitive than other lymphocyte subsets[27–30].

Complementary approaches to detect cell apoptosis may include evaluation of several cellular aspects, such as caspase activation, cell morphology and mitochondrial status[31]. If apoptosis was occurring in irradiated macrophages, effector caspases, those responsible for the apoptosis execution phase, such as caspase-7, should be functionally activated, this is proteolytically cleaved[31]. Therefore, caspase-7 expression was evaluated by western blot analysis, using an antibody which detects both the full length caspase-7 (35 kDa) and the large fragment of cleaved caspase-7 (20 kDa) (Fig. 1C). We demonstrated that there was no caspase-7 cleavage in irradiated macrophages. Additionally and focusing on the strongest cumulative dose (10 Gy) (6 h time point) (Supplementary Fig. S1), we confirmed, by using a positive control, that caspase-7 was not being cleaved in irradiated macrophages. At the same time, we also evaluated the cleavage of caspase-3, which is is a critical executioner of apoptosis[31], and the cleavage of PARP, a substrate of several cell death proteases[32], including caspase-3 (Supplementary Fig. S1). The antibody which detects the full length caspase-3 (35 kDa) also recognizes the large fragment of cleaved caspase-3 (17 kDa). Positive controls for caspase-3 and -7 activation as well as for PARP cleavage are presented in this western blot panel. As demonstrated by membrane overexposure, no cleavage of caspase-3/–7 nor even of PARP were observed in irradiated macrophages, suggesting that apoptosis was not occurring.

In order to identify apoptosis-related morphological features in macrophages after exposure to cumulative ionizing radiation doses, cells were observed and photographed after each irradiation dose. No apoptotic signs, such as cell shrinkage, pyknosis or loss of membrane integrity[31], were observed in 10 Gy irradiated and non-irradiated macrophages, suggesting that both populations were similarly viable (Fig. 1D).

To complement previous observations, macrophage mitochondrial function was evaluated 20 h after exposure to 2, 6 and 10 Gy cumulative doses, using the resazurin reduction assay (Fig. 1E). Results evidenced that, independently of the dose, ionizing radiation did not affect macrophage metabolic activity.

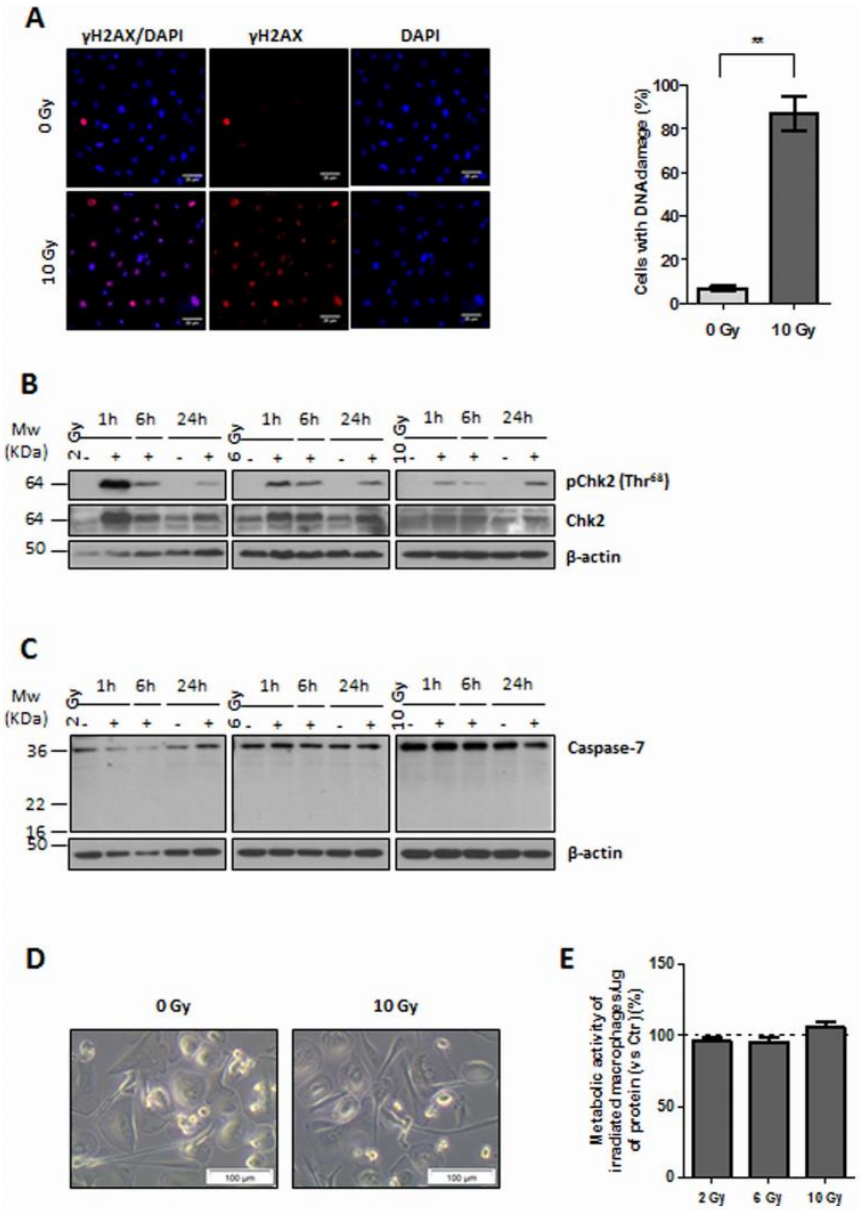

Figure 1. Irradiated human monocyte-derived macrophages are viable and metabolically active, despite DNA damage. (**A**) Radiation-induced macrophage DNA damage is demonstrated by immunocytochemistry for Ser139-phosphorylated H2AX (γH2AX) (red), while nuclei were counterstained with DAPI (blue). Scale bar represents 20 μm. Graph indicates the percentage of macrophages ($n = 3$ and 400 cells/donor counted) exhibiting γH2AX foci. Data was analysed with paired *t*-test. $^{**}P < 0.01$. (**B**) Western blot analysis of total and phosphorylated Chk2 (Thr68) expression on non-irradiated (−) or irradiated (2, 6 and 10 Gy) (+) macrophages ($n = 3$), upon 1, 6 and 24 h. (**C**) Western blot analysis of caspase-7 expression on non-irradiated (−) or irradiated (2, 6 and 10 Gy) (+) macrophages ($n = 3$), upon 1, 6 and 24 h. In all Western blots β-actin was used as loading control. (**D**) Brightfield microscopic images of non-irradiated (0 Gy) and irradiated macrophages (10 Gy). Scale bar represents 100 μm. (**E**) Quantification of the metabolic activity of irradiated macrophages (2, 6 or 10 Gy) ($n = 8$), normalized to the activity of non-irradiated ones, and expressed as percentage. One-sample *t*-test was performed.

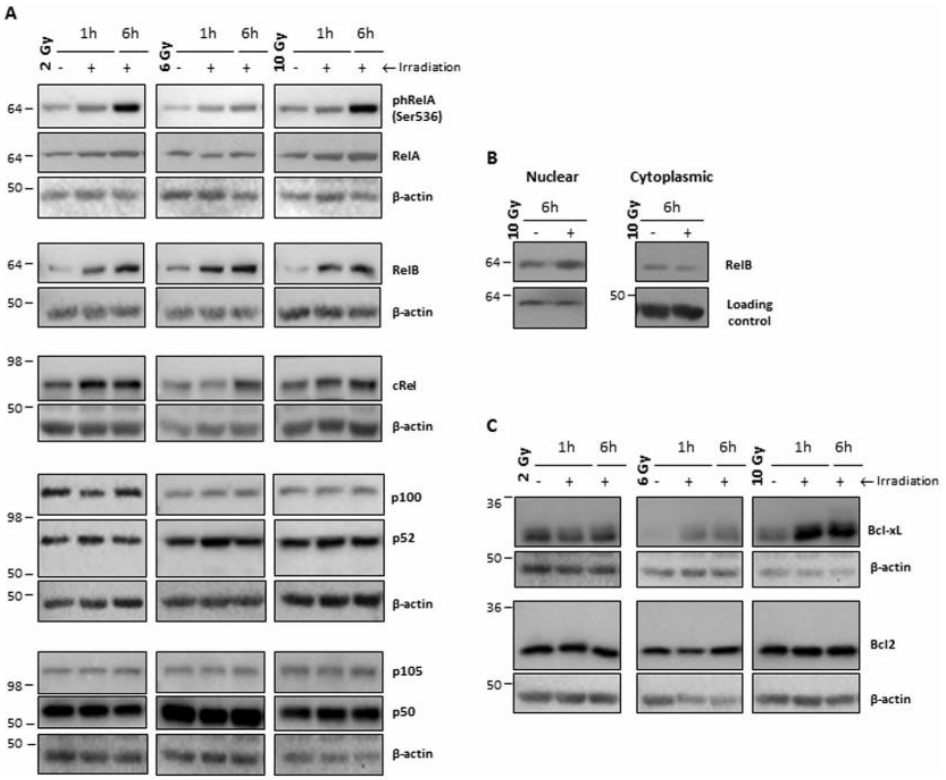

Figure 2. Ionizing radiation induces macrophage NF-κB activation and increases Bcl-xL expression. (**A**) Evaluation of RelA phosphorylation (Ser536) and RelB, cRel, p100/p52 and p105/p50 subunit expression, 1 and 6 h after irradiation (2, 6 and 10 Gy). (**B**) RelB nuclear translocation 6 h after macrophage irradiation (10 Gy). Histone deacetylase 1 (HDAC1) and β-actin were used as loading controls for nuclear and cytoplasmic fractions, respectively. (**C**) Evaluation of Bcl2 and Bcl-xL expression after macrophage irradiation. Western blot images are representative of protein expression/phosphorylation status in distinct donors (at least $n = 4$), evaluated in two independent experiments.

Overall, we concluded that irradiated macrophages are viable and metabolically active, and do not activate apoptosis, despite radiation-induced DNA damage.

Ionizing radiation activates macrophage pro-survival and NF-κB signalling pathway. Considering macrophage survival following ionizing radiation exposure, a typical radiation-induced survival pathway mediated by NF-κB[33], was next investigated. Western blot analysis for the five NF-κB family subunits, RelA, RelB, cRel, p52/p100 and p50/p105 (Fig. 2A) as well as for IκBα (Supplementary Fig. S2), a NF-κB inhibitor, were performed. Densitometry analysis (Supplementary Fig. S3) confirmed that ionizing radiation increased the expression of RelB at every time points in every donors and tend to slightly increase cRel expression (Supplementary Fig. S3A). Additionally, ionizing radiation also tend to slightly increase p100 processing, as suggested by a reduced p52/p100 ratio at 2 and 6 Gy. No major alterations were found in phRel/RelA and p105/p50 levels. Moreover, alterations in RelB suggested that ionizing radiation upregulates macrophage NF-κB, mainly through the non-canonical pathway. As RelB was the most consistently upregulated subunit in response to radiation, we next confirmed RelB nuclear translocation, by evaluating its expression, in nuclear and cytoplasmic extracts, 6 h after 10 Gy cumulative dose (Fig. 2B). In fact, ionizing radiation increased RelB nuclear expression (Supplementary Fig. S3B), suggesting its nuclear translocation and subsequent activation. As NF-κB can induce the expression of anti-apoptotic proteins, such as Bcl2 and Bcl-xL[34], we evaluated the expression of these targets after macrophage irradiation (2, 6 and 10 Gy) (Fig. 2C). The most relevant alteration was the Bcl-xL increased expression, particularly at 10 Gy (Supplementary Fig. S3C). The increase was visible at 1 h and sustained after 6 h, suggesting induction of macrophage pro-survival activity. Since Bcl-xL maximum expression tends to occur after 10 Gy, all subsequent experiments were performed with macrophages submitted to this cumulative ionizing radiation dose.

Ionizing radiation induces a reduction in anti-inflammatory macrophage phenotype. To evaluate the effect of ionizing radiation on macrophage polarization profile, we characterized the pattern of expression of pro- or anti-inflammatory cytokines/chemokines and cell surface receptors, by a combination of quantitative

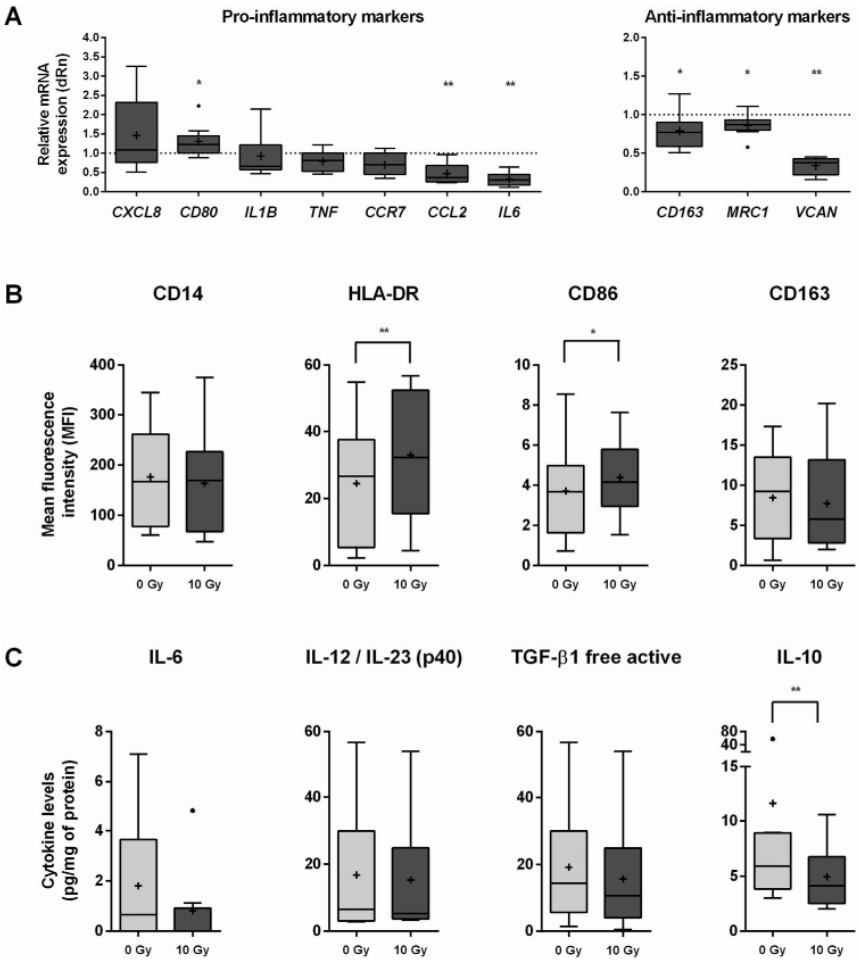

Figure 3. Irradiated macrophages present a reduced anti-inflammatory phenotype. (**A**) mRNA expression profile of pro-(*CXCL8*, *CD80*, *IL1B*, *TNF*, *CCR7*, *CCL2*, *IL6*) and anti-inflammatory (*CD163*, *MRC1*, *VCAN*) macrophage markers, by real-time PCR, 20 h after 10 Gy. Graphs represent mRNA expression of irradiated macrophages compared to non-irradiated ones (dotted line) (at least $n = 7$ per marker). β-actin was used as housekeeping gene. Wilcoxon signed rank test was used to compare the median of each dataset against a hypothetical median value of 1. (**B**) Expression of a monocyte/macrophage lineage (CD14), pro-(HLA-DR and CD86) and anti-inflammatory (CD163) macrophage markers was determined, by flow cytometry, 20 h after irradiation (at least $n = 6$ per marker). Paired *t*-test was used for statistical analysis. (**C**) Levels of pro-(IL-6, IL-12/IL-23(p40)) and anti-inflammatory (TGF-β1 and IL-10) cytokines were determined in macrophage CM ($n = 9$) by ELISA, 20 h after irradiation. Data was normalized to protein concentration. Wilcoxon matched pair test was used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$. Median is represented by the horizontal line inside the box plots, while the average is indicated by a "+". In IL-6 and IL-10 graphics outliers are also indicated.

real-time PCR, flow cytometry and ELISA. Ionizing radiation induced a significant increase ($P < 0.05$) of *CD80* expression, but not of other pro-inflammatory gene markers. In fact, *IL6* and *CCL2* were significantly downregulated ($P < 0.01$) after macrophage irradiation (Fig. 3A). Furthermore, all anti-inflammatory gene markers tested, *CD163*, *MRC1* and *VCAN* were significantly downregulated ($P < 0.05$ or $P < 0.01$) upon irradiation (Fig. 3A). To investigate how these changes could translate into cell surface receptor expression, flow cytometry was then performed. Results evidenced that ionizing radiation significantly increased HLA-DR ($P < 0.01$) and CD86 ($P < 0.05$) (macrophage pro-inflammatory markers), and tend to decrease CD163 (macrophage anti-inflammatory marker), but did not alter the expression of the monocyte/macrophage lineage marker CD14 (Fig. 3B). To further complete the polarization profile analysis, levels of pro-(IL-6 and IL-12/IL-23(p40)) and anti-inflammatory (TGF-β1 and IL-10) cytokines were evaluated by ELISA, using conditioned medium (CM) from non-irradiated (0 Gy) and irradiated (10 Gy) macrophages, normalized to protein concentration (Fig. 3C). Interestingly, only IL-10 levels were significantly downregulated ($P < 0.01$) by ionizing radiation. IFN-γ and TNF-α levels were also investigated, but

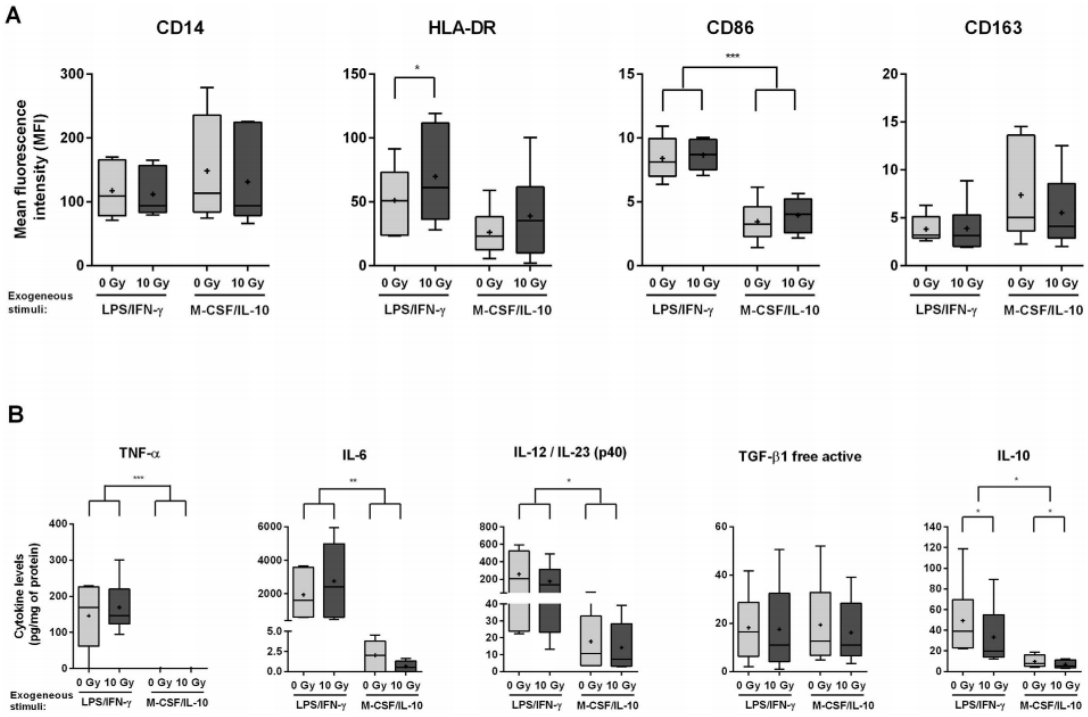

Figure 4. Upon exogenous stimulation, irradiated macrophages polarize towards a pro- or an anti-inflammatory phenotype. Non-irradiated and 10 Gy irradiated macrophages ($n = 6$) were stimulated, for 20 h, with LPS (100 ng/ml) and IFN-γ (20 ng/ml) towards a pro-inflammatory, or with M-CSF (10 ng/ml) and IL-10 (20 ng/ml) towards an anti-inflammatory phenotype. (**A**) Expression of monocyte/macrophage lineage (CD14), pro-(HLA-DR and CD86) and anti-inflammatory (CD163) macrophage markers was determined by flow cytometry. (**B**) Macrophage CM levels of pro-(TNF-α, IL-6, IL-12/IL-23(p40)) and anti-inflammatory (TGF-β1 and IL-10) cytokines were determined by ELISA. Data was normalized to protein concentration. Paired *t*-test and one-way ANOVA were used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Median is represented by the horizontal line inside the box plots, while the average is indicated by a "+".

were undetectable for both macrophage populations. Altogether, this data suggests that ionizing radiation directs macrophages towards a reduced anti-inflammatory phenotype, without achieving however a complete classical pro-inflammatory profile.

Upon exogenous stimulation, irradiated macrophages polarize towards pro- or anti-inflammatory phenotypes. Unstimulated M-CSF differentiated macrophages typically exhibit a more anti-inflammatory-like phenotype, but they are able to polarize towards a pro- or an anti-inflammatory phenotype, after proper exogenous stimulation[35,36]. The functional response to external stimuli, termed plasticity, is one of the major hallmarks of the mononuclear phagocyte system[37]. To assess how ionizing radiation affects macrophage plasticity, we evaluated whether irradiated macrophages were still able to polarize towards a pro- or an anti-inflammatory phenotype, upon proper exogenous stimulation. Therefore, upon 10 Gy cumulative dose exposure, macrophages were stimulated for 20 h with LPS (100 ng/mL) and IFN-γ (20 ng/mL), towards a pro-inflammatory phenotype, or with M-CSF (10 ng/mL) and IL-10 (20 ng/mL), towards an anti-inflammatory one. The expression of pro- and anti-inflammatory cell surface markers and cytokines/chemokines was evaluated by flow cytometry (Fig. 4A) and ELISA (Fig. 4B), respectively. Comparison between exogenously stimulated (LPS/IFN-γ or M-CSF/IL-10) non-irradiated (0 Gy) and irradiated (10 Gy) macrophages indicated that ionizing radiation did not affect macrophage ability to polarize towards a pro- or an anti-inflammatory phenotype, upon proper exogenous stimuli. LPS/IFN-γ-stimulated macrophages (both non-irradiated and irradiated) present higher levels of pro-inflammatory markers such as HLA-DR, CD86, TNF-α, IL-6 and IL-12/IL-23 (p40), being the last four significantly different (from $P < 0.05$ to $P < 0.001$), when compared to M-CSF/IL-10-stimulated macrophages (Fig. 4A,B). On the other side, LPS/IFN-γ-stimulated macrophages tend to present lower levels of the anti-inflammatory marker CD163, comparing to M-CSF/IL-10-stimulated macrophages (Fig. 4A). Our results evidenced that, contrary to expectations, the pro-inflammatory stimuli (LPS/IFN-γ) also induced higher levels of immunosuppressive IL-10 cytokine than the anti-inflammatory ones (M-CSF/IL-10). However, this phenomenon was also previously reported in M-CSF-conditioned dendritic cell precursors, which exhibit a rapid IL-10 release upon LPS stimulation and may therefore participate in the modulation of inflammation and immune response[38].

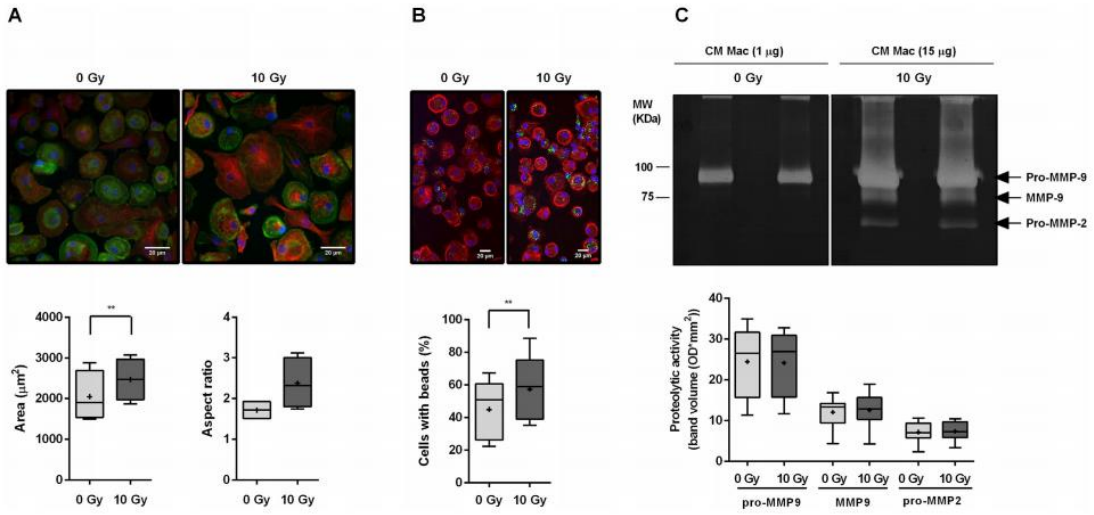

Figure 5. Ionizing radiation increases macrophage area, aspect ratio and phagocytic rate, but does not alter MMP-2 and -9 activities. (**A**) Actin (green) and tubulin (red) stainings of non-irradiated and 10 Gy irradiated macrophages ($n = 4$). Quantification of cell area and aspect ratio was performed using Fiji software. Scale bar indicates 20 µm. (**B**) Phagocytic ability of non-irradiated and 10 Gy-irradiated macrophages ($n = 5$) was determined after 1 h incubation with FITC-labelled (green) *Staphylococcus aureus* particles. F-actin was stained with rhodamine phalloidin (red). The percentage of macrophages able to phagocyte *S.aureus* particles was quantified using Fiji software. Scale bar indicates 20 µm. (**C**) MMP-2 and -9 activity was evaluated by gelatin zymography, using 1 and 15 µg of protein from CM of non-irradiated and 10 Gy-irradiated macrophages ($n = 10$). White bands of proteolytic activity were revealed on a Coomassie Blue-stained gelatin gel. All data was analysed with paired *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$. Median is represented by the horizontal line inside the box plots, while the average is indicated by a "+".

Interestingly, irradiated macrophages stimulated with LPS/IFN-γ presented significantly ($P < 0.05$) higher levels of HLA-DR, when compared to their non-irradiated counterparts (Fig. 4A). On the other hand, under stimulation with M-CSF/IL-10, irradiated macrophages tend to exhibit lower CD163 and higher HLA-DR levels, when compared to non-irradiated macrophages (Fig. 4A,B). Also, irradiated macrophages polarized towards a pro- or an anti-inflammatory phenotype presented a significant decrease ($P < 0.05$) in IL-10 levels, when compared to non-irradiated and also exogenous stimulated ones (Fig. 4B). Altogether, these results demonstrate that, upon exogenous stimulation, irradiated macrophages are still able to polarize towards a pro- or an anti-inflammatory phenotype. However, ionizing radiation by itself tends to promote the pro-inflammatory phenotype.

Ionizing radiation induces macrophage morphological alterations and increases their phagocytic rate, without altering MMP-2 and MMP-9 activities. In order to characterize morphological changes, macrophage area and aspect ratio were determined 1 h after exposure to 10 Gy (Fig. 5A). Results indicated that irradiated macrophages presented a significantly higher ($P < 0.05$) cell area ($2{,}471 \pm 513.3\ \mu m^2$) and tend to increase cell aspect ratio (quotient between major and minor cell axes) (2.38 ± 0.63), when compared to non-irradiated ones (area $2{,}047 \pm 616.3\ \mu m^2$ and aspect ratio 1.72 ± 0.24).

Besides functional and morphological plasticity, other features like phagocytic ability and proteolysis are also characteristic of macrophages[39]. To evaluate the effect of ionizing radiation on macrophage phagocytic ability, non-irradiated and 10 Gy irradiated macrophages were incubated, for 1 h, with FITC-labelled and killed *Staphylococcus (S.) aureus* particles. Results revealed that ionizing radiation significantly increased ($P < 0.01$) the percentage of macrophages able to phagocyte *S. aureus* particles (Fig. 5B).

The impact of ionizing radiation on the activity of MMP-2 and MMP-9, two matrix metalloproteases involved in the promotion of cancer cell invasion and angiogenesis[40], was determined by gelatin zymography. Using 1 µg of protein from macrophage CM, no differences in pro-MMP-9 proteolytic activity were found, between non-irradiated and 10 Gy irradiated macrophages (Fig. 5C). The same conclusion was extended to MMP-9 and pro-MMP-2 proteolytic bands, when 15 µg of macrophage CM protein were loaded. Our results evidence that ionizing radiation induces macrophage morphologic alterations and increases their phagocytic rate, without affecting macrophage MMP-2 and MMP-9 proteolytic activities.

Irradiated macrophages promote cancer cell invasion and angiogenesis. To investigate the effect of ionizing radiation on macrophage pro-invasive cancer cell activity, non-irradiated or 10 Gy irradiated macrophages and irradiated colorectal cancer cells were confronted on Matrigel invasion assays (Fig. 6A). Irradiated as non-irradiated macrophages significantly promoted ($P < 0.001$ or $P < 0.01$) the invasion of irradiated RKO

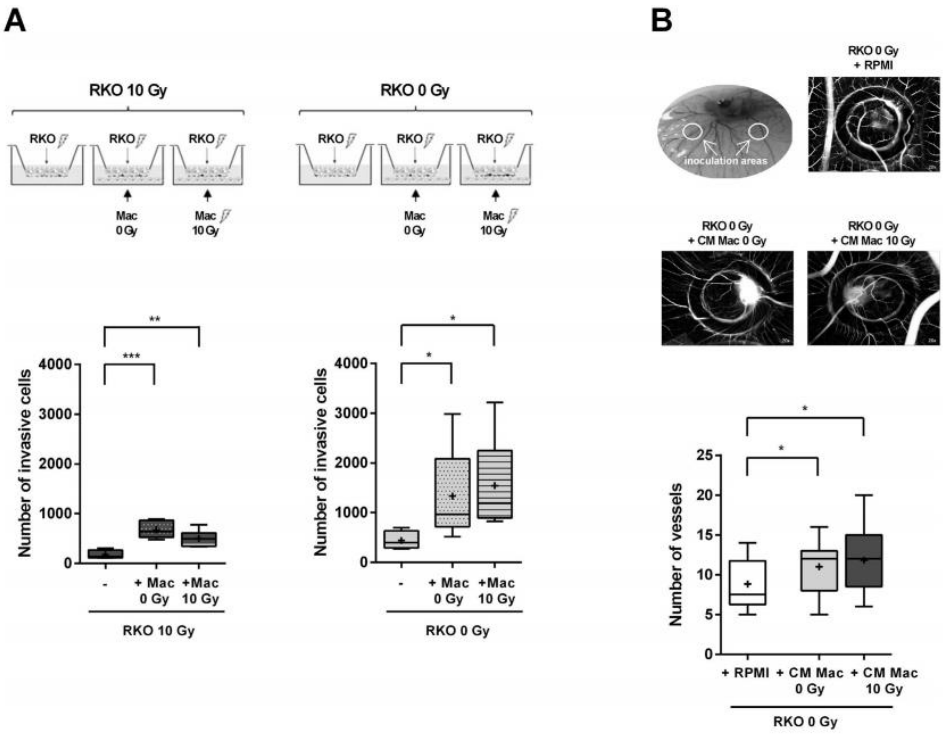

Figure 6. Ionizing radiation does not affect macrophage ability to promote cancer cell invasion and cancer cell-mediated angiogenesis. (**A**) Matrigel invasion assays were established confronting RKO cells (upper compartment) and macrophages (lower compartment) after being separately exposed or not to 10 Gy cumulative ionizing radiation dose. The six possible combinations are represented in the scheme. Invasive cells were counterstained with DAPI and counted on the microscope. (**B**) RKO cells were inoculated, for 72 h, with CM from non-irradiated or 10 Gy irradiated macrophages, in rings (inoculation areas), on top of CAM. The comparison of RKO+ CM Mac 0 Gy versus RKO+CM Mac 10 Gy was evaluated in two rings within the same fertilized egg ($n = 18$), while the condition RKO+RPMI was performed in a single distinct egg ($n = 16$). Analysis of RKO-induced angiogenesis was performed through quantification of the number of new vessels in control and experimental conditions. ANOVA analysis demonstrated a significant difference between groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The median is represented by the horizontal line inside the box plots, while the average is indicated by a "+".

cells. To clarify if macrophage pro-invasive ability was dependent on the presence of irradiated colorectal cancer cells, we used non-irradiated RKO cells. Herein, we verified the same effect previously observed indicating that irradiated macrophages do not lack the ability to promote cancer cell invasion. Moreover, we also observed that irradiation of RKO cells alone significantly decrease ($P < 0.05$) its invasive potential. We could indeed predict that RKO cell invasive potential was going to be highly affected by radiation exposure due to the RKO intrinsic radiosensitivity, previously reported in the literature. An accumulation of about 70% of RKO cells was described in G2/M-phase 16 ± 24 h after 12 Gy irradiation in pH 7.5 medium[41]. RKO cells were also included in a group of radiosensitive cells, upon evaluation of the clonogenic survival of 27 human tumour cell lines after ionizing radiation exposure[42].

In order to investigate the effect of irradiated macrophages on cancer cell-induced angiogenesis, a chick embryo chorioallantoic membrane (CAM) assay was performed. CM from both non-irradiated and 10 Gy irradiated macrophages significantly promoted ($P < 0.05$) RKO-induced angiogenesis (Fig. 6B). No differences were observed between RKO cells treated with CM derived from non-irradiated and irradiated macrophages. Altogether, Matrigel invasion and CAM-based assays demonstrated that, in the present experimental context, ionizing radiation *per se* was not able to restrain macrophages' endogenous ability to promote cancer cell invasion and cancer cell–induced angiogenesis, which constitute two main hallmarks of cancer[3].

Discussion

The present work aims to understand the effect of ionizing radiation on human macrophages, as they are important components of the tumour microenvironment, and also highly recruited into tumours during radiation therapy[43]. The recurrent use of mouse models and lack of clinically relevant doses in other studies have not allowed a full understanding of this effect[16–20]. In the present study, we characterized, for the first time, the response of human primary macrophages to cumulative ionizing radiation doses, using the same fractionated scheme as used during

cancer patients' treatment (2 Gy/fraction/day), up to 10 Gy cumulative dose. As a model, we used M-CSF-cultured macrophages, differentiated from peripheral-blood monocytes, as it is considered the predominant *in vitro* system to study human tissue macrophages[44]. M-CSF is a growth factor involved in the recruitment of monocytes/macrophages to tissues and also in the regulation of macrophage function within tumours[45]. Taking into account that the cellular radiation response is a complex process[46], several features like DNA damage, NF-κB signalling pathway, polarization profile, plasticity, phagocytosis, proteolysis and the ability to promote cancer cell activities, were evaluated in irradiated human macrophages.

Macrophage resistance to ionizing radiation was first reported in mouse models some decades ago[47,48]. Nowadays, it is recognized that human macrophages, similarly to regulatory T cells (Tregs), dendritic cells, Natural Killer (NK) cells and thrombocytes, as well as MDSCs, display a more radiation resistant phenotype than other immune cell populations, such as monocytes[27,29,30,46]. However, most of the studies were performed in mouse macrophages irradiated with X-ray doses, which barely mimic the fractionated scheme used in cancer patients' treatment. Our results demonstrated that irradiated macrophages exhibited higher DNA damage, confirmed through increased H2AX phosphorylation, than non-irradiated ones. DNA damage induced by ionizing radiation is known to lead to Chk2-specific phosphorylation (Thr68) at sites of DSBs[49]. In agreement, we demonstrated that Chk2 phosphorylation increased along time and according to the exposure doses. Despite DNA damage, irradiated macrophages remain viable and metabolically active, leading to understand which survival pathways were activated. We focused on NF-κB signalling, which is known to induce a pro-survival response in cells exposed to single 2 Gy doses[33,50]. Our results demonstrated that RelB expression was consistently increased after macrophage exposure to 2, 6 and 10 Gy cumulative doses. RelB nuclear translocation suggested the involvement of the non-canonical NF-κB pathway in radiation-induced macrophage response. Our data also demonstrated an increased expression of the anti-apoptotic Bcl-xL protein, which also contributes to the promotion of pro-survival activity. Interestingly, a recent study demonstrated that ionizing radiation induces RelB to activate Bcl-xL in cancer cells[51].

NF-κB transcription factors are not only important for cell survival upon irradiation, but are also considered major regulators of inflammation processes and, particularly in macrophages, their activation is required for the anti- to pro-inflammatory phenotype transition[52–54]. Radiation-induced NF-κB alterations in macrophages led to the characterization of macrophage inflammatory status upon irradiation, which data was summarized in the scheme of Fig. 7. In the present study, 10 Gy cumulative ionizing radiation dose significantly decreased both anti-inflammatory gene markers (*CD163*, *MRC1*, *VCAN*) and the immunosuppressive cytokine IL-10, and increased HLA-DR and CD86 expression of M-CSF differentiated macrophages (Fig. 7). Additionally, we also demonstrated that bacterial phagocytosis, a classical feature of pro-inflammatory macrophages, was found to be significantly increased in irradiated macrophages. In fact, mouse macrophages subjected to 8 Gy single dose were also described to slightly enhance phagocytosis of inert latex beads[55]. Together, our data supports the hypothesis that ionizing radiation may drive macrophages towards a pro-inflammatory phenotype. Although irradiated macrophages exhibited increased *CD80* expression, other classical pro-inflammatory markers, like *IL1B*, *TNF* and *IL6* were unaltered, downregulated, or undetectable at the cytokine level. This suggests that irradiated macrophages did not reach a classical pro-inflammatory phenotype, despite the observed reduction of their original anti-inflammatory-like phenotype. The mechanism behind this shift in macrophage phenotype may indeed rely on radiation-induced NF-κB alterations, particularly in RelB subunit as its expression is increased in irradiated macrophages. In fact, very recently, a switch of anti-inflammatory to pro-inflammatory macrophages was found to be directly mediated by RelB induction in M-CSF and TNF-stimulated osteoclast precursors[56]. However, further experiments are still required to confirm RelB involvement in macrophage anti-inflammatory phenotype reduction, suggested to occur after macrophage ionizing radiation exposure.

To simplify the understanding of our hypothesis, we represented the typical pro- and anti-inflammatory macrophage profiles as two extremes of a continuous polarization spectrum (generally represented as a line). However, macrophage polarization has been revealed as a very complex and dynamic system and new representations are emerging as long as new knowledge in this area is coming out. Recently, Ruffell and Coussens summarized the macrophage polarization system as a circle, which does not consider pro- and anti-inflammatory macrophage profiles, but rather emphasize that macrophage functional roles (angiogenesis, cytotoxicity, stimulation, suppression or chemotaxis) (included in an inner circle) are dictated by the integration of multiple stimuli (represented in an outer circle)[57]. Therefore, we may speculate that ionizing radiation exposure could also constitute one of the distinct stimuli capable of polarizing macrophages into a different phenotype, which does not completely corresponds to a pro- or an anti-inflammatory one, but could rather include features from both phenotypes, as we demonstrated in this work, or even from other phenotypes still to be defined. Overall, our study defines, for the first time, a molecular profile for human macrophages subjected to cumulative ionizing radiation doses, emphasizing the important role of fractionated ionizing radiation doses, as used in radiotherapy, to direct macrophages towards a pro-inflammatory phenotype, which is recognized to be tumour cytotoxic[37]. According to the literature, the relation between ionizing radiation exposure and inflammatory response seems to be dependent not only on the cell type analysed and radiation quality, but mainly on the delivered dose[58]. Low doses (maximum of 12 Gy at $\leq$1.0 Gy/fraction), usually applied in non-malignant disorders or received by normal tissues outside the tumour target volume, induce an anti-inflammatory phenotype, while higher doses (single doses $\geq$2 Gy, total doses $\geq$40 Gy) are reported to have a pro-inflammatory effect[59,60]. Most of the studies, aiming to reveal the role of irradiation on macrophage inflammatory status, are performed using *in vitro* or *ex vivo* mouse macrophages. Although mouse models have widely contributed to our understanding of ionizing radiation-induced effect on macrophages, it is also well recognized that mouse and human macrophages present many distinct features[61]. Particularly, mouse macrophages are high producers of nitric oxide (NO) and L-citrulline from L-arginine, via inducible nitric oxide synthase (iNOS) activation, while NOS and arginase activities in human macrophages are quite debatable[62,63]. These considerations should be taken into account when extrapolating data from one species to another.

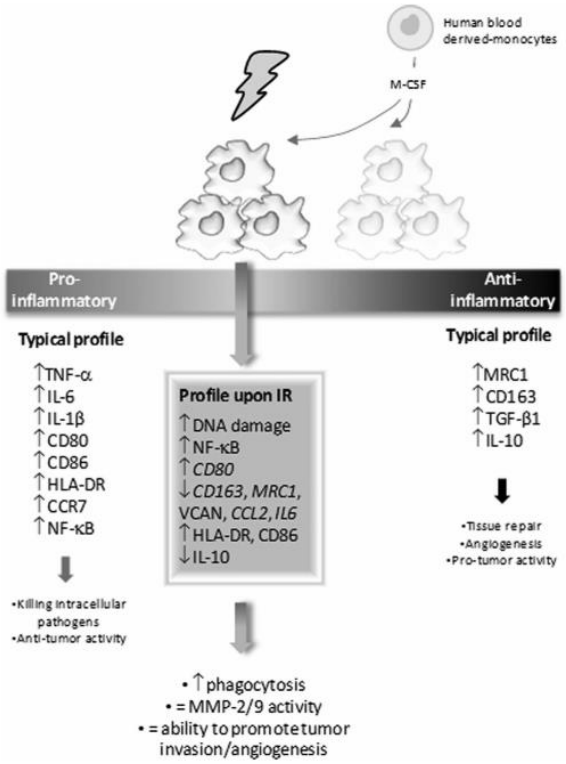

Figure 7. Schematic representation of the effect of ionizing radiation on human blood monocyte-derived macrophages. Two main macrophage functional polarization status are recognized: a pro-inflammatory, responsible for killing intracellular pathogens and antitumour activity, and an anti-inflammatory one, which induces tissue repair, angiogenesis and promotes tumour activity. Pro-inflammatory macrophages produce high levels of TNF-α, IL-6 and IL-1β cytokines and exhibit CD80, CD86, HLA-DR, CCR7 and NF-κB increased expression, while anti-inflammatory ones express CD163, MRC1 and produce high levels of TGF-β1 and IL-10 cytokines. In the present study, we demonstrated that irradiated macrophages exhibit a decrease of anti-inflammatory (*CD163*, *MRC1* and IL-10) and an increase of other pro-inflammatory (*CD80*, CD86, HLA-DR) markers. Although irradiated macrophages are more effective than non-irradiated ones at phagocytosis, a typical feature of pro-inflammatory macrophages, they fail to reach a classical pro-inflammatory phenotype, as they do not produce high levels of TNF-α, IL-6, IL-1β and CCR7. On the other hand, and similarly to their counterparts, irradiated macrophages are able to promote cancer cell invasion and cancer cell-induced angiogenesis. Our data suggests that M-CSF differentiated macrophages, exposed to cumulative ionizing radiation doses up to 10 Gy, exhibit a reduced anti-inflammatory-like phenotype, compared to non-irradiated ones, probably moving towards a pro-inflammatory phenotype. However, although irradiated macrophages exhibit characteristics from both pro- and anti-inflammatory phenotypes, they do not perfectly match to any of these typical profiles, appearing to acquire intermediate characteristics.

We have also demonstrated that irradiated macrophages are as able as their non-irradiated counterparts to promote RKO cancer cell invasion and cancer cell-induced angiogenesis. This is the first report demonstrating that, at least during the first week (5 days) of radiotherapy, human macrophages sustain its ability to promote cancer cell invasion and cancer cell-induced angiogenesis, which is a matter of concern. Nevertheless, we need to be cautious when extrapolating this data to the clinic, as during further neoadjuvant treatment the situation may change. Not only because cell response along treatment time may be different but also because *in vivo* other host cells and several environmental factors may contribute to tumour response to radiotherapy. As our team previously demonstrated, MMP activity is known to be an important factor for macrophage-mediated cancer cell invasion[64]. Therefore, the sustained promotion of cancer cell invasion by irradiated macrophages may be associated with the fact that MMP-2 and MMP-9 activity is not being affected by radiation exposure. Furthermore, we also showed that irradiated macrophages are still able to promote cancer cell-induced angiogenesis.

In summary, our work adds valuable data on characterization of a plethora of functions in human macrophages, subjected to cumulative ionizing radiation doses, as used during cancer patients' treatment, which were not addressed before. We have characterized important aspects like plasticity, proteolysis, phagocytosis and cancer cell activity promotion. We demonstrated that human macrophages subjected to cumulative doses of ionizing radiation are viable, metabolically active and exhibit increased survival signalling, through NF-κB activation and increased

Bcl-xL expression, despite DNA damage. Irradiated macrophages also present a reduced anti-inflammatory profile, increased phagocytosis and unaltered MMP-2 and -9-mediated proteolysis. Pro-inflammatory-like macrophages are known to be cytotoxic and exhibit antitumoural activities and may therefore contribute to the efficacy of local radiotherapy[37]. Our data also demonstrates that irradiation maintains macrophage ability to promote cancer cell invasion and cancer cell-induced angiogenesis. Overall, although radiotherapy mainly induces cancer cell death, other components of the microenvironment, particularly macrophages, are also irradiated and could persist still sustaining the activity of residual radioresistant cancer cells. Furthermore, this knowledge opens new perspectives for macrophage clinical targeting, prior, after or concomitantly to ionizing radiation, as a strategy to improve radiotherapy efficacy.

Material and methods

Ethics statement. In the present study, human monocytes were obtained from buffy coats, which are a highly leukocyte-enriched waste-product that results from a whole blood donation, from healthy blood donors. A collaboration protocol between our Institution and Centro Hospitalar São João (CHSJ), where blood donations of Portugal North region are performed, allows the use of these products for investigation purposes. All studies using this human material were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. Informed consent was obtained from all subjects before each blood donation.

Human monocyte isolation and macrophage differentiation. Human monocytes were isolated as previously described[64]. Following this procedure, over 80% of isolated monocytes were found to be CD14-positive[64]. For monocyte-macrophage differentiation, 1.2×10^6 cells/9.6 cm^2 (6-well plate) were cultured in complete RPMI1640 medium with GlutaMax (Invitrogen) in the presence of 50 ng/mL of macrophage colony-stimulating factor (M-CSF) (ImmunoTools). Culture medium (1.5 mL/well) was replaced after one week and macrophage differentiation was completed 13 days after monocyte isolation, as at this stage macrophages were shown to provide a higher stimulus for cancer-cell invasion, than with shorter differentiation times[64].

Cell culture. RKO (CRL-2577) cells, derived from a human colon carcinoma, were purchased from the American Type Culture Collection (ATCC). Cells were maintained at 37 °C, 5% CO_2 humidified-atmosphere, in RPMI1640 (L-Glutamine) (Invitrogen) supplemented with 10% FBS (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

Ionizing radiation exposure. Prior irradiation, a dosimetry plan was established (ELEKTA CMS XiO v.4.7.0). Culture plates were submitted to a Computerized Tomography (CT) scan and the volume occupied by two entire plates was defined as the target volume. Two beam fields, one anterior-posterior and other posterior-anterior, were arranged to deliver 2 Gy per fraction to this target volume. Inside the defined volume, the total dose varied from 198 cGy to 202 cGy. As the 4 cGy difference was not significant, the same dose was considered homogenously distributed through plates. To guarantee this uniform dose and to avoid the build-up region of the 18 MV photon beam, 5 water plates were added above, and 5 below the culture plates during irradiation. Medium was renewed before the first irradiation. Both macrophages and RKO cells were then exposed to 1–5 cumulative ionizing radiation doses (2 Gy/fraction/day), for a week (Monday to Friday). Therefore, the maximum cumulative irradiation dose, equivalent to 5 fractions, totalized 10 Gy (Supplementary Fig. S4). Photon beam was produced by a PRIMUS (Siemens, Malvern, PA, USA) linear particle accelerator, used for human radiotherapy sessions, operated at 18 MV at the Radiotherapy Service of CHSJ. To avoid differences between non-irradiated and irradiated cells, caused by medium agitation during transport to/from the Radiotherapy Service, control cells were also transported, but were not radiation-exposed.

Cell viability. For a proper follow-up of macrophage during irradiation week, cells were carefully observed under a light microscope (Olympus) and daily pictures were taken. To complement this qualitative data, macrophage metabolic activity was determined through resazurin reduction assay, which was considered a sensitive, reproducible and non-destructive assay to measure cell response to irradiation[65]. Briefly, 20 h after irradiation (2, 6 or 10 Gy), macrophages were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich) for 3 h at 37 °C and 5% CO_2. Fluorescence intensity was measured (530 nm Ex/590 nm Em), using the multi-mode microplate reader Synergy MX (BioTek) and values were normalized to protein concentration in the CM, measured with detergent-compatible (DC) protein assay (BioRad). Data from irradiated macrophages was then compared with the respective controls and expressed as percentage.

Protein extraction and Western Blot. Whole cell protein-extracts were performed 1, 6 and 24 h after irradiation (2, 6 and 10 Gy) ($n = 4$), using lysis buffer supplemented with a cocktail of proteases/phosphatases inhibitors, as previously described[64]. Nuclear/cytoplasmic extracts were performed 6 h after 10 Gy ($n = 5$), using appropriate lysis buffer [10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 1 mM DTT, 0.1% Igepal, protease/phosphatase inhibitors cocktail]. Cytoplasmic extracts were obtained after centrifugation at 14 000 rpm for 10 min, at 4 °C. For nuclear extracts, pellets were resuspended in another lysis buffer [20 mM Hepes pH 7.9 , 420 mM KCl , 1.5 mM $MgCl_2$, 1 mM DTT, 25% Glycerol, protease/phosphatase inhibitors cocktail], rocked for 15 min at 4 °C, centrifuged at 14 000 rpm for 15 min, and sonicated. Following SDS-PAGE, gels were transferred onto polyinylidine difluoride (PVDF) membrane, which were then incubated, for 1 h, with primary antibodies against the following proteins: p105/p50, p100/p52 (Millipore), Bcl-xL (BD Biosciences), phospho-IκBα (Ser32/36) (clone 5A5), IκBα (clone 44D4), phospho-RelA (Ser536) (clone 93H1), Bcl-2 (clone 50E3), phospho-Chk2 (Thr68), Chk2, caspase-3, caspase-7, cleaved PARP (clone D64E10) (Cell Signalling), RelA, RelB,

cRel (Santa Cruz Biotechnology). Positive controls for caspase-3 and -7 activation as well as for PARP cleavage were used. Antibody against β-actin (clone 8H10D10) (Cell Signalling) was used to normalize protein expression. Goat anti-rabbit or horse anti-mouse-Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signalling) were used for 1 h, followed by ECL-Detection (Thermo Fisher Scientific). Densitometry analysis of western blot images from Fig. 2 was performed with Quantity One software (BioRad).

RNA extraction, cDNA preparation and quantitative PCR analysis. Total RNA, from non-irradiated or 10 Gy irradiated macrophages, was extracted using TriPure Isolation Reagent (Roche), according to manufacturer's instructions. RNA was converted to cDNA using 150 U of SuperScript™ II Reverse Transcriptase, 1× first strand buffer, 10 mM DTT 0.1 M (Invitrogen), 0.5 mM dNTPs 10 mM (Bioron), 8U of rRNasin (Promega) and RNase/DNase free water (Gibco). To evaluate mRNA expression levels of pro- and anti-inflammatory gene markers, quantitative PCR using Brilliant II Sybr green kit (Stratagene/Agilent Technologies) and specific MX3005P 96-well semi-skirted plates, were performed. Samples were analysed on the MX3005P qPCR platform (Stratagene/Agilent). The following primers, from Invitrogen, were used for RT-qPCR: CXCL8, F: 5′-CCAGGAAGAAACCACCGGA-3′, R: 5′-GAAATCAGGAAGGCTGCCAAG-3′; IL1B, F: 5′-GGCAGGGAACCAGCATC-3′, R: 5′-CCGACCACCACTACAGCAA-3′; TNF F: 5′-GGCTGGAGCTGAGAGATA-3′, R: 5′-CAGCCTTGGCCCTTGAAGA-3′. Primer sets for *ACTB* (used as a normalizing gene), *CXCL12* and *CCL2* were obtained from Qiagen, while probes for CD80, CCR7, IL6, CD163, MRC1 and VCAN were from Applied Biosystems.

Macrophage polarization. After 10 Gy cumulative ionizing radiation exposure, macrophages were stimulated, during 20 h, with 100 ng/mL LPS (Sigma-Aldrich) plus 20 ng/mL IFN-γ (Immunotools) towards a pro-inflammatory phenotype (M1-like), or with 10 ng/mL M-CSF plus 20 ng/mL IL-10 (Immunotools) towards an anti-inflammatory (M2-like) one[44].

Flow cytometry. For cell surface receptor expression analysis, non-irradiated and 10 Gy irradiated macrophages, subjected or not to further cytokine-induced polarization, as above detailed, were kept on ice, washed with PBS, gently detached by scraping and resuspended in FACs buffer [PBS, 2% FBS (Lonza), 0.01% sodium azide]. Stainings with anti-human CD14-APC (clone MEM-18), HLA-DR-PE (MEM-12), CD86-FITC (clone BU63) (Immunotools) and CD163-PE (clone GHI/61) (R&D Systems) antibodies were performed in the dark for 30 min. After additional washing steps, macrophages were fixed for 15 min in 4% paraformaldehyde (PFA). Isotype-matched antibodies were used as negative controls, to define background staining. Cells were acquired on a FACS Calibur™ Flow Cytometer (BD Biosciences), using Cell Quest Software (collecting 1×10^4 cells). Analysis was performed with FlowJo software (v7.6.5). Mean fluorescent intensity was calculated by subtracting the respective isotype control intensity.

Enzyme-linked immunosorbent assay (ELISA). IL-6, IL-12/IL-23(p40), TNF-α, TGF-β1 free active and IL-10 cytokine levels were determined, according to manufacturer's instructions (BioLegend), in CM from non-irradiated and 10 Gy irradiated macrophages, subjected or not to further cytokine-induced polarization as above detailed. Briefly, 50 μL of cell culture supernatant were added to a 96-well plate pre-coated with the capture antibody of interest. The soluble proteins bound to the capture antibody were detected using a biotinylated antibody, followed by an avidin-HRP conjugated solution. Finally, the addition of TMB substrate, resulted in a colour change, which intensity was proportional to the amount of antigen captured. Absorbance was then read at 450 and 570 nm. Cytokine levels were determined by plotting values on a standard curve and normalizing them to CM protein concentration.

Immunocytochemistry. DNA damage and morphology were evaluated, by immunocytochemistry, in non-irradiated and 10 Gy irradiated macrophages (4×10^4). After 1 h, macrophages were fixed with 4% PFA for 20 min and immunocytochemistry procedure was then performed as previously described[64]. Macrophages were incubated with monoclonal antibodies for phosphorylated histone-H2AX (Ser139) (γH2AX) (clone JBW301) (Millipore) or α-tubulin (Sigma-Aldrich), for 1 h, followed by goat-anti mouse AlexaFluor-594-conjugated-secondary antibody (Invitrogen) incubation, for 45 min in the dark. F-actin was stained for 15 min with 0.5 μM Phalloidin-FITC (Sigma-Aldrich), 0.1 M EGTA and 1 M $MgSO_4$. Finally, nucleus was stained with 10 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) solution. Multiwell plate-based screening was performed with a Leica DMI6000 B inverted motorized fluorescence microscope (Leica Microsystems). Microscopic images are represented at 300× magnification.

Phagocytosis. Ready-made pHrodo green *Staphylococcus aureus* BioParticles Conjugate (1 μm diameter) (Invitrogen) were resuspended in PBS up to 1 mg/mL and gently vortexed and sonicated for homogenous dispersion. Non-irradiated and 10 Gy irradiated macrophages (4×10^4) were then incubated with 1.6×10^6 *S. aureus* particles at 37 °C and 5% CO_2, to evaluate phagocytic activity. After 1 h, macrophages were washed in PBS and fixed with 4% PFA for 20 min. For cell identification, F-actin was stained with rhodamine-labeled phalloidin (1:100 dilution) (Invitrogen) for 30 min, after previous permeabilization with 0.2% Triton X-100 and 5% bovine serum albumin (BSA) blocking. Finally, for nuclei visualization, macrophages were incubated with 10 μg/mL DAPI solution for 5 min. Multiwell plate-based screening was performed with IN Cell Analyzer 2000 (GE Healthcare). Microscopic images are represented at 150× magnification. The number of cells able to phagocyte *S. aureus* particles was then determined, using Fiji software[66].

Gelatin zymography. Macrophage CM (1 and 15 μg of protein), collected 24 h after 10 Gy cumulative ionizing radiation exposure, were used to evaluate MMP-2 and MMP-9 activities, through gelatin-zymography, as previously described[64].

Matrigel invasion assays. To evaluate macrophage-mediated RKO cell invasion, non-irradiated or 10 Gy irradiated RKO cells (5×10^4) were seeded on the upper compartment of Matrigel-coated inserts of 8-μm pore size (BD Biosciences), while non-irradiated or 10 Gy irradiated macrophages (2×10^5), were added on the bottom, for 24 h, as previously described[64].

Chick embryo *in vivo* angiogenesis assay. The chick embryo CAM model was used to evaluate RKO-induced angiogenic response in the presence of macrophage CM. Therefore, commercially available fertilized chick (*Gallus gallus*) eggs were horizontally incubated at 37.5 °C, in a humidified atmosphere. On embryonic development day (EDD)3, a square window was opened in the shell after removal of 1.5–2 mL of albumen, to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and eggs re-incubated. On EDD10, RKO cells (1×10^6) resuspended in CM from non-irradiated or 10 Gy irradiated macrophages were placed on top of the same CAM, into two independent 3 mm silicone rings, under sterile conditions. For control, RKO cells resuspended in RPMI medium were inoculated in a different egg. Eggs were re-sealed and returned to the incubator for additional 72 h. On EDD13, rings were removed, the CAM was excised from embryos and photographed *ex-ovo* under a stereoscope, using a 20× magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (<20 μm diameter) growing radially towards the inoculation area was counted in a blind fashion.

Statistical analysis. All graphs and statistical analysis were performed using GraphPad Prism Software v5 (GraphPad-trial version). Data was analysed for Gaussian distribution using the D'Agostino and Pearson normality test, when $n \geq 8$. To test the hypothesis that irradiated macrophages are different from non-irradiated ones, Wilcoxon matched pairs test was used for non-parametric samples, while *t*-test (either paired *t*-test or one sample *t*-test) was used for parametric data or when $n < 8$. For other comparisons, one-way ANOVA test was performed. Statistical significance was achieved when $P < 0.05$.

References

1. Delaney, G., Jacob, S., Featherstone, C. & Barton, M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer* **104,** 1129–1137 (2005).
2. Ringborg, U. *et al.* The Swedish Council on Technology Assessment in Health Care (SBU) systematic overview of radiotherapy for cancer including a prospective survey of radiotherapy practice in Sweden 2001–summary and conclusions. *Acta Oncol* **42,** 357–365 (2003).
3. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–674 (2011).
4. Bernier, J., Hall, E. J. & Giaccia, A. Radiation oncology: a century of achievements. *Nat Rev Cancer* **4,** 737–747 (2004).
5. Allen, B. J., Bezak, E. & Marcu, L. G. Quo vadis radiotherapy? Technological advances and the rising problems in cancer management. *Biomed Res Int* **2013,** 749203 (2013).
6. Begg, A. C., Stewart, F. A. & Vens, C. Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* **11,** 239–253 (2011).
7. Madani, I., De Neve, W. & Mareel, M. Does ionizing radiation stimulate cancer invasion and metastasis? *Bull Cancer* **95,** 292–300 (2008).
8. Hofmeister, V., Schrama, D. & Becker, J. C. Anti-cancer therapies targeting the tumor stroma. *Cancer Immunol Immunother* **57,** 1–17 (2008).
9. Di Caro, G., Marchesi, F., Laghi, L. & Grizzi, F. Immune cells: plastic players along colorectal cancer progression. *J Cell Mol Med* **17,** 1088–1095 (2013).
10. Jain, R. K. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. *J Clin Oncol* **31,** 2205–2218 (2013).
11. Lauber, K., Ernst, A., Orth, M., Herrmann, M. & Belka, C. Dying cell clearance and its impact on the outcome of tumor radiotherapy. *Front Oncol* **2,** 116 (2012).
12. Condeelis, J. & Pollard, J. W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124,** 263–266 (2006).
13. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat Rev Cancer* **9,** 239–252 (2009).
14. Vacchelli, E. *et al.* Trial Watch: Anticancer radioimmunotherapy. *Oncoimmunology* **2,** e25595 (2013).
15. Hellevik, T. & Martinez-Zubiaurre, I. Radiotherapy and the Tumor Stroma: The Importance of Dose and Fractionation. *Front Oncol* **4,** 1 (2014).
16. UNSCEAR. Biological mechanisms of radiation actions at low doses. A white paper to guide the Scientific Committee's future programme of work. (United Nations 2012).
17. College, O. in *College Physics* Vol. 3 (ed OpenStax College) Ch. 32 (2012).
18. Frischholz, B. *et al.* Reduced secretion of the inflammatory cytokine IL-1beta by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation. *Autoimmunity* **46,** 323–328 (2013).
19. Tsukimoto, M., Homma, T., Mutou, Y. & Kojima, S. 0.5 Gy gamma radiation suppresses production of TNF-alpha through up-regulation of MKP-1 in mouse macrophage RAW264.7 cells. *Radiat Res* **171,** 219–224 (2009).
20. Wunderlich, R. *et al.* Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. *Clin Exp Immunol* **179,** 50–61 (2015).
21. Mah, L. J., El-Osta, A. & Karagiannis, T. C. gammaH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia* **24,** 679–686 (2010).
22. Sulli, G., Di Micco, R. & d'Adda di Fagagna, F. Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. *Nat Rev Cancer* **12,** 709–720 (2012).
23. Radford, I. R., Murphy, T. K., Radley, J. M. & Ellis, S. L. Radiation response of mouse lymphoid and myeloid cell lines. Part II. Apoptotic death is shown by all lines examined. *Int J Radiat Biol* **65,** 217–227 (1994).
24. Eriksson, D. & Stigbrand, T. Radiation-induced cell death mechanisms. *Tumour Biol* **31,** 363–372 (2010).
25. Seita, J. & Weissman, I. L. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* **2,** 640–653 (2010).

26. Youn, J. I. & Gabrilovich, D. I. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur J Immunol* **40,** 2969–2975 (2010).
27. Kozin, S. V. *et al.* Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation. *Cancer Res* **70,** 5679–5685 (2010).
28. Xu, J. *et al.* CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer Res* **73,** 2782–2794 (2013).
29. Vatner, R. E. & Formenti, S. C. Myeloid-derived cells in tumors: effects of radiation. *Semin Radiat Oncol* **25,** 18–27 (2015).
30. Barker, H. E., Paget, J. T., Khan, A. A. & Harrington, K. J. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer* **15,** 409–425 (2015).
31. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35,** 495–516 (2007).
32. Chaitanya, G. V., Steven, A. J. & Babu, P. P. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell Commun Signal* **8,** 31 (2010).
33. Rashi-Elkeles, S. *et al.* Parallel induction of ATM-dependent pro- and antiapoptotic signals in response to ionizing radiation in murine lymphoid tissue. *Oncogene* **25,** 1584–1592 (2006).
34. Tamatani, M. *et al.* Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. *J Biol Chem* **274,** 8531–8538 (1999).
35. Jaguin, M., Houlbert, N., Fardel, O. & Lecureur, V. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol* **281,** 51–61 (2013).
36. Martinez, F. O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* **177,** 7303–7311 (2006).
37. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25,** 677–686 (2004).
38. Kwan, W. H., Boix, C., Gougelet, N., Fridman, W. H. & Mueller, C. G. LPS induces rapid IL-10 release by M-CSF-conditioned tolerogenic dendritic cell precursors. *J Leukoc Biol* **82,** 133–141 (2007).
39. Burke, B. & Lewis, C. E. *The Macrophage*. 2nd edn (Oxford University Press, 2002).
40. Deryugina, E. I. & Quigley, J. P. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* **25,** 9–34 (2006).
41. Park, H. J., Lyons, J. C., Ohtsubo, T. & Song, C. W. Cell cycle progression and apoptosis after irradiation in an acidic environment. *Cell Death Differ* **7,** 729–738 (2000).
42. Williams, J. R. *et al.* Overview of radiosensitivity of human tumor cells to low-dose-rate irradiation. *Int J Radiat Oncol Biol Phys* **72,** 909–917 (2008).
43. Shiao, S. L. & Coussens, L. M. The tumor-immune microenvironment and response to radiation therapy. *J Mammary Gland Biol Neoplasia* **15,** 411–421 (2010).
44. Murray, P. J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41,** 14–20 (2014).
45. Lin, E. Y., Nguyen, A. V., Russell, R. G. & Pollard, J. W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193,** 727–740 (2001).
46. Heylmann, D., Rodel, F., Kindler, T. & Kaina, B. Radiation sensitivity of human and murine peripheral blood lymphocytes, stem and progenitor cells. *Biochim Biophys Acta* **1846,** 121–129 (2014).
47. McLennan, G., Oberley, L. W. & Autor, A. P. The role of oxygen-derived free radicals in radiation-induced damage and death of nondividing eucaryotic cells. *Radiat Res* **84,** 122–132 (1980).
48. Perkins, E. H., Nettesheim, P. & Morita, T. Radioresistance of the engulfing and degradative capacities of peritoneal phagocytes to kiloroentgen x-ray doses. *J Reticuloendothel Soc* **3,** 71–82 (1966).
49. Ward, I. M., Wu, X. & Chen, J. Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *J Biol Chem* **276,** 47755–47758 (2001).
50. Brach, M. A. *et al.* Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. *J Clin Invest* **88,** 691–695 (1991).
51. Zhu, L. *et al.* RelB regulates Bcl-xl expression and the irradiation-induced apoptosis of murine prostate cancer cells. *Biomed Rep* **2,** 354–358 (2014).
52. Hagemann, T., Biswas, S. K., Lawrence, T., Sica, A. & Lewis, C. E. Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. *Blood* **113,** 3139–3146 (2009).
53. Biswas, S. K. *et al.* A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* **107,** 2112–2122 (2006).
54. Lawrence, T. & Natoli, G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol* **11,** 750–761 (2011).
55. Conrad, S., Ritter, S., Fournier, C. & Nixdorff, K. Differential effects of irradiation with carbon ions and x-rays on macrophage function. *J Radiat Res* **50,** 223–231 (2009).
56. Zhao, Z. *et al.* TNF Induction of NF-kappaB RelB Enhances RANKL-Induced Osteoclastogenesis by Promoting Inflammatory Macrophage Differentiation but also Limits It through Suppression of NFATc1 Expression. *PLoS One* **10,** e0135728 (2015).
57. Ruffell, B. & Coussens, L. M. Macrophages and therapeutic resistance in cancer. *Cancer Cell* **27,** 462–472 (2015).
58. Rodel, F. *et al.* Immunomodulatory properties and molecular effects in inflammatory diseases of low-dose x-irradiation. *Front Oncol* **2,** 120 (2012).
59. Seegenschmiedt, M. H., Makoski, H. B., Trott, K. R. & Brady, L. W. E. *Radiotherapy for Non-Malignant Disorders*. (Springer Verlag 2008).
60. Rodel, F., Frey, B., Multhoff, G. & Gaipl, U. Contribution of the immune system to bystander and non-targeted effects of ionizing radiation. *Cancer Lett* **356,** 105–113 (2015).
61. Mestas, J. & Hughes, C. C. Of mice and not men: differences between mouse and human immunology. *J Immunol* **172,** 2731–2738 (2004).
62. Schneemann, M. *et al.* Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. *J Infect Dis* **167,** 1358–1363 (1993).
63. Thomas, A. C. & Mattila, J. T. "Of mice and men": arginine metabolism in macrophages. *Front Immunol* **5,** 479 (2014).
64. Cardoso, A. P. *et al.* Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. *Oncogene* **33,** 2123–2133 (2014).
65. Anoopkumar-Dukie, S. *et al.* Resazurin assay of radiation response in cultured cells. *Br J Radiol* **78,** 945–947 (2005).
66. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9,** 676–682 (2012).

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Author Contributions

A.T.P. designed the study, performed the experiments and drafted the manuscript. M.L.P. and A.P.C. contributed to experimental planning, protocol optimization and *in vitro* experiments. C.M. helped with macrophage polarization experiments. M.T.P. carried out *in vivo* C.A.M. assay experiments. A.F.M. and P.C. provided technical support on image acquisition and analysis. R.F., A.M. and M.M. elaborated the dosimetric planning, managed the irradiation schedule and provided clinical input. S.G.S. helped in the acquisition and interpretation of macrophage polarization data. M.M., R.S., M.A.B. and S.R. provided scientific input and reviewed the manuscript. M.J.O. supervised the study data and drafted the manuscript. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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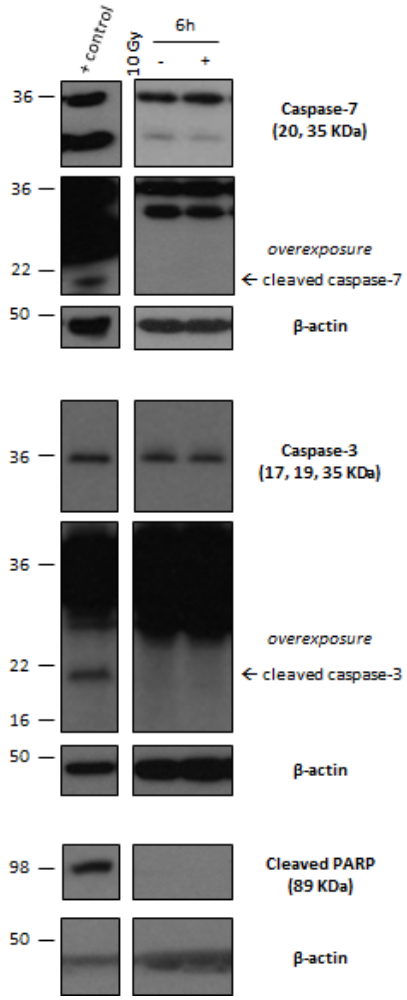
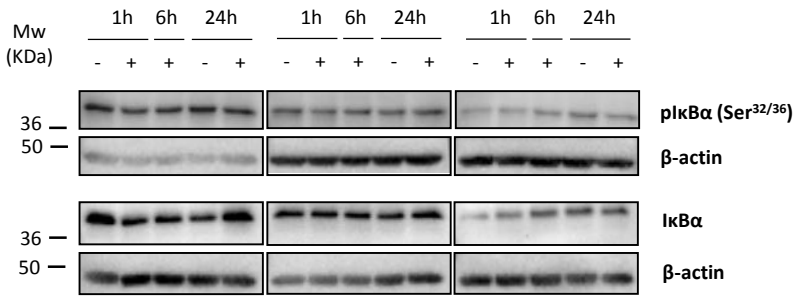
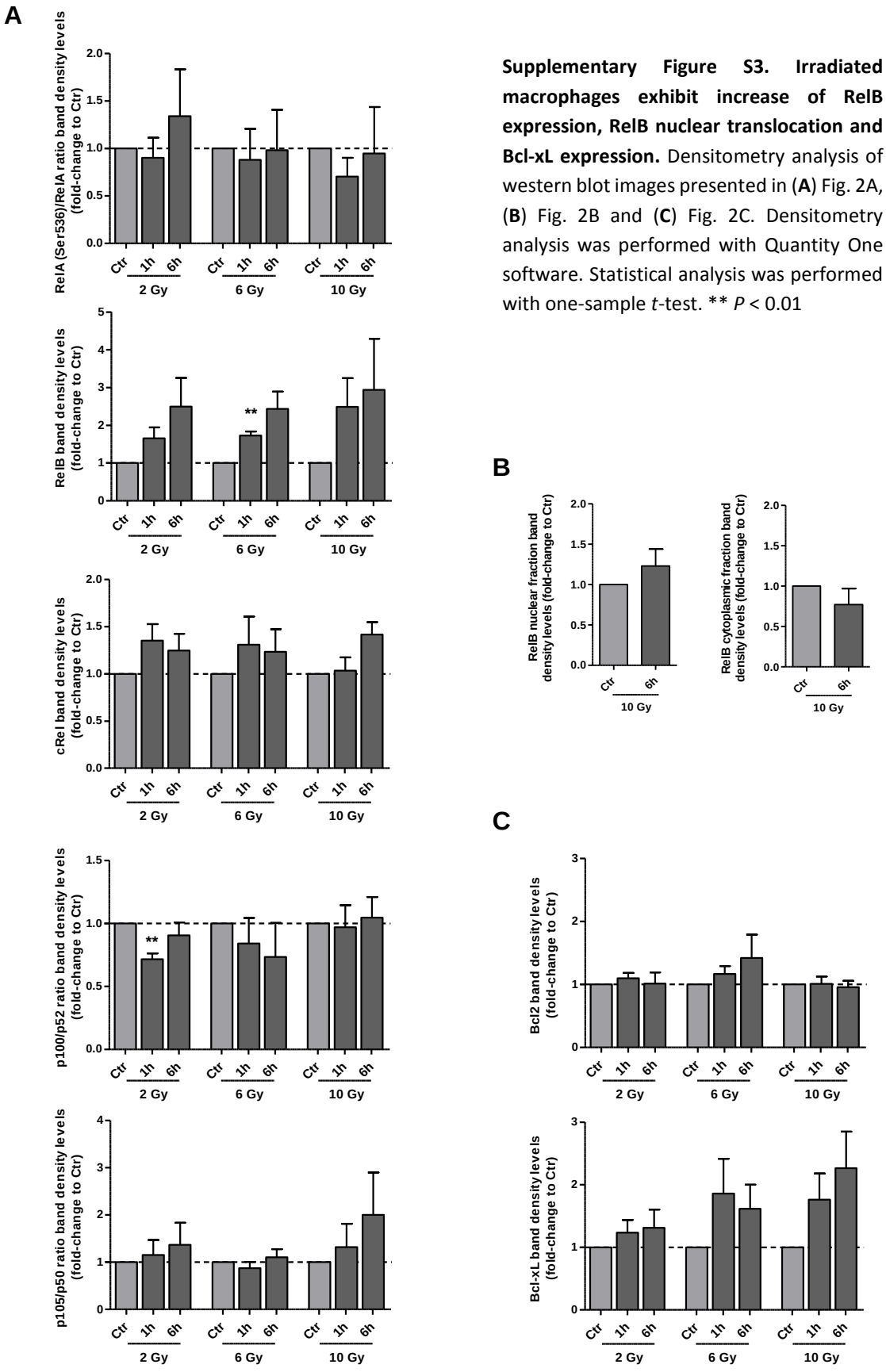
Supplemental file



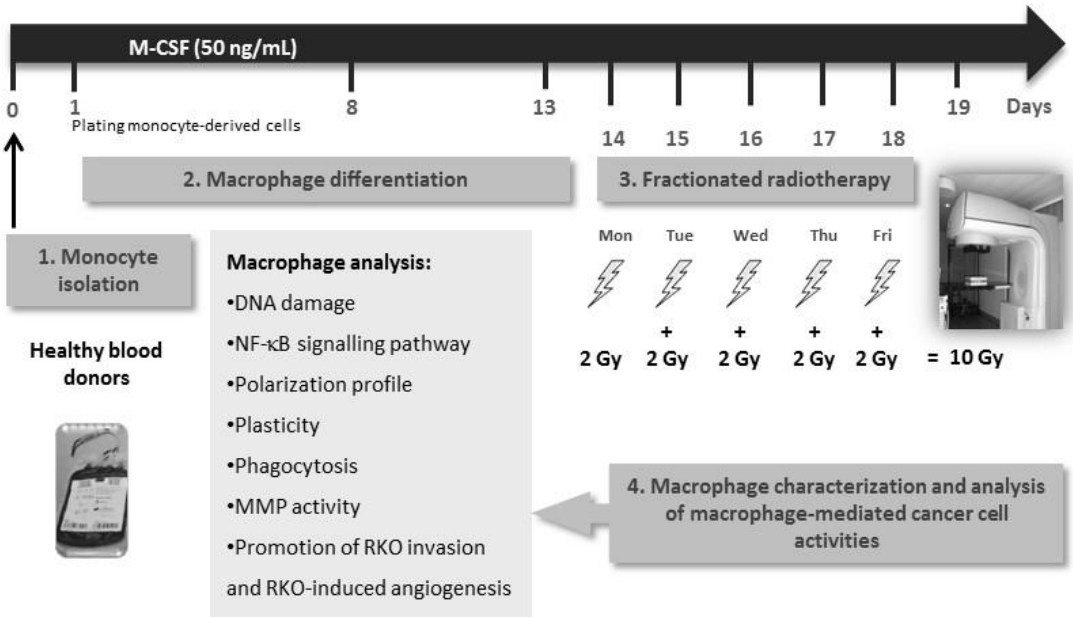
Supplementary Figure S1. Irradiated macrophages do not exhibit caspase-3/-7 nor PARP cleavage. Expression levels of total caspase-3, caspase-7 and cleaved PARP were evaluated by western blot analysis (*n* = 4) in macrophages 6 h after 10 Gy cumulative dose exposure. β-actin was used as loading control. Positive controls for caspase-3/-7 and PARP cleavage are also present.



Supplementary Figure S2. Irradiated macrophages do not exhibit phosphorylation of IκBα. Evaluation of total and phosphorylated IκBα (Ser32/36) levels, by western blot analysis in macrophages 1, 6 and 24 h after 2, 6 and 10 Gy cumulative doses (*n* = 4).



Supplementary Figure S3. Irradiated macrophages exhibit increase of RelB expression, RelB nuclear translocation and Bcl-xL expression. Densitometry analysis of western blot images presented in (**A**) Fig. 2A, (**B**) Fig. 2B and (**C**) Fig. 2C. Densitometry analysis was performed with Quantity One software. Statistical analysis was performed with one-sample *t*-test. ** $P < 0.01$



Supplementary Figure S4. Schematic overview of the methodology used in this work. Monocytes were isolated from human healthy blood donors' buffy coats, seeded at day 1, and cultured in the presence of 50 ng/mL macrophage colony-stimulating factor (M-CSF) to allow their differentiation. Upon thirteen days, macrophages and RKO colorectal cancer cells were then X-ray irradiated with daily doses of 2 Gy for 5 days. Upon 10 Gy of cumulative ionizing radiation dose, the direct effect of ionizing radiation on macrophages and on macrophage-mediated RKO cell activities were evaluated, as listed.

Complementary unpublished results

Exploring the NF-κB signalling in irradiated macrophages

Aiming to complement some of the NF-κB signalling alterations in irradiated macrophages previously described in our manuscript, the following additional results are presented herein. In the manuscript, we evaluated the expression of some pro-inflammatory molecules that were also NF-κB targets, namely *IL1B* and *CCL2*, which were found to be downregulated in macrophages 24 h after exposure to 10 Gy cumulative ionizing radiation dose. We asked whether that reduction occurred at an earlier time-point and cumulative dose. Therefore, the expression of *IL1B*, *CCL2* and also of another pro-inflammatory molecule not described in the manuscript, *CCL5*, was evaluated 1 h after exposure to 2, 6 and 10 Gy cumulative doses (Figure C1).

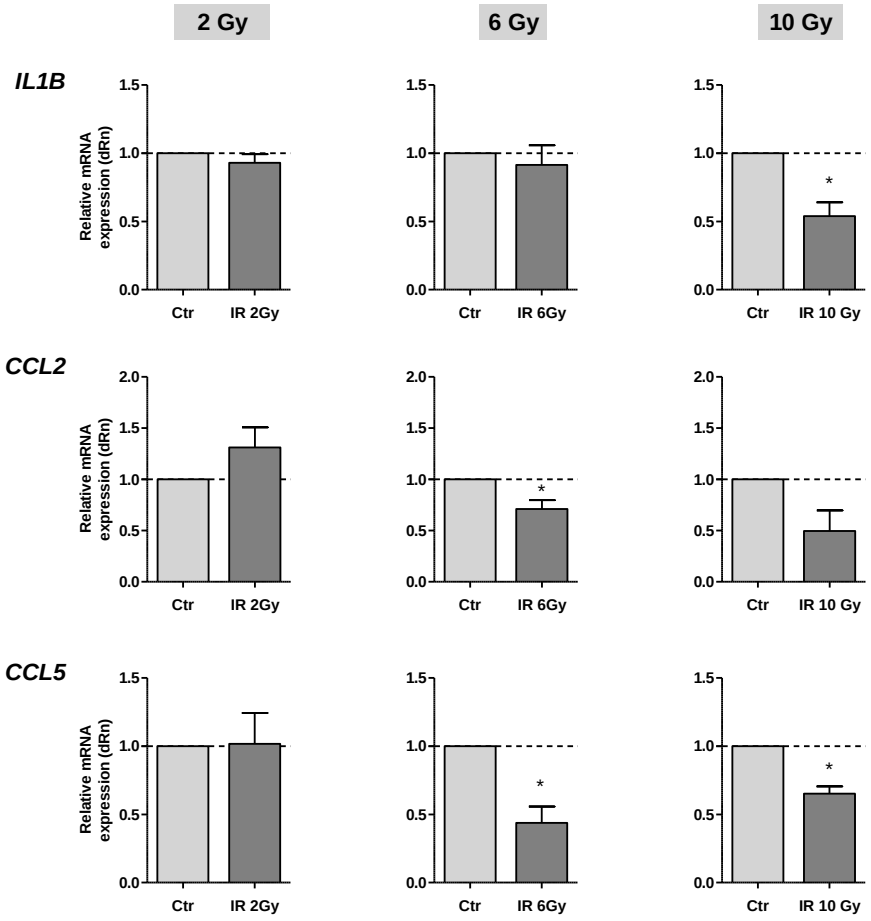


Figure C1 – mRNA expression of *IL1B*, *CCL2* and *CCL5* NF-κB targets. The expression of *IL1B, CCL2* and *CCL5* was evaluated in macrophages (n = 4) 1 h after exposure to 2, 6 or 10 Gy. One sample t-test was used for statistical purposes. *$P < 0.05$.

Results demonstrated that *IL1B* was significantly downregulated already at 1 h after exposure to 10 Gy, but not at earlier cumulative ionizing radiation doses, while *CCL2* and *CCL5* were downregulated since exposure to 6 Gy.

From the manuscript, a lack of statistical significance of mRNA expression levels of another pro-inflammatory molecule, *CXCL8*, suggested no expression alterations in macrophages 24 h after exposure to 10 Gy cumulative ionizing radiation. However, knowing that *CXCL8* is a classical NF-κB target and that NF-κB was activated in irradiated macrophages, we asked whether *CXCL8* mRNA expression could be altered at different doses and time-points and explored it at 1 and 6 h after macrophage exposure to 2, 6 and 10 Gy (Figure C2).

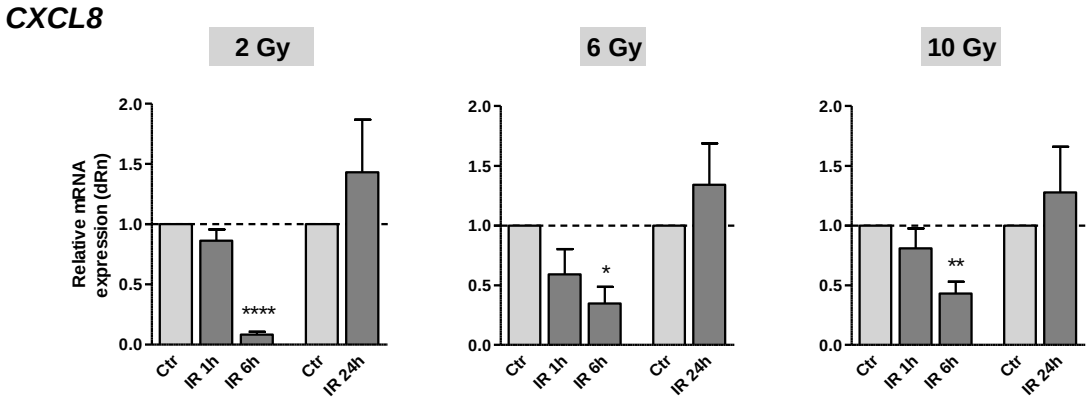


Figure C2 – mRNA expression of *CXCL8* in macrophages exposed to 2, 6 and 10 Gy cumulative ionizing radiation doses. The expression of *CXCL8* was evaluated in macrophages (n = 4) 1, 6 and 24 h after exposure to 2, 6 or 10 Gy. One sample t-test was used for statistical purposes. *P < 0.05, **P < 0.01, ****P < 0.0001.

Our results demonstrate that there was a significant reduction of *CXCL8* expression 6 h after macrophage exposure to 2, 6 and 10 cumulative ionizing radiation doses. Although at 24 h there was a tendency to increase *CXCL8* levels, this was not consistent in every blood donors, contrarily to what was verified at 6 h after irradiation. In fact, at 24 h after 10 Gy, macrophages from two blood donors clearly upregulated *CXCL8* mRNA expression, while the other two downregulated it. This may suggest an individual adaptation regarding radiation exposure, which may be dependent on blood donor features (such as gender, age or even the anti-inflammatory drugs taken before blood donation that may interfere with response of immune cells to external stimuli). Finally, these results evidence the different kinetics of *CXCL8* expression upon macrophage irradiation, reinforcing the careful required when interpreting data from a single time-point after radiation exposure.

Our manuscript also demonstrated that macrophages exposed to 10 Gy cumulative ionizing radiation dose exhibit reduced levels of released IL-10. As IL-10 is an anti-inflammatory cytokine also targeted by NF-κB, we explored whether its reduction occurred at earlier cumulative doses (Figure C3).

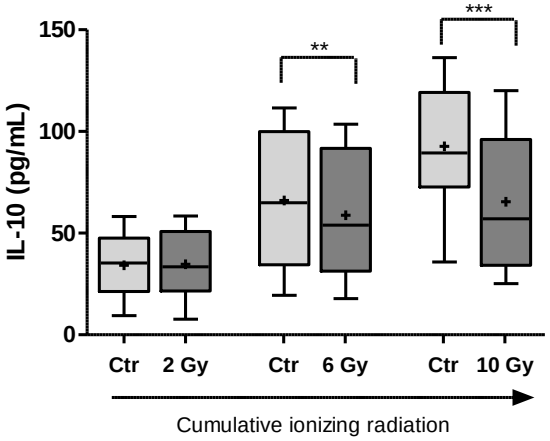


Figure C3 – Cumulative ionizing radiation doses decrease macrophage IL-10 levels. CM from macrophages was collected 24 h after exposure to 2 (*n* = 9), 6 (*n* = 6) and 10 (*n* = 10) Gy and IL-10 levels were quantified by ELISA. Of note, IL-10 levels were not normalized for protein concentration in the CM. "+" indicates the mean. Paired *t*-test was used for statistical purposes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results demonstrated that irradiated macrophages exhibited reduced IL-10 levels, not only after exposure to 10 Gy, but already at 6 Gy cumulative ionizing radiation dose.

Although an increased expression of NF-κB targets might be expected, from the NF-κB activation previously described in irradiated macrophages, the expression of *IL1B*, *CCL2*, *CCL5*, *CXCL8* and IL-10, was found to be downregulated in macrophages at a specific time-point after radiation exposure. This downregulation could be due to a positive or negative regulation of NF-κB target genes by the specific NF-κB dimers activated upon macrophage irradiation.

Due to the importance of NF-κB signalling in radioresistance, we aimed to evaluate whether NF-κB inhibition interfered directly with macrophage response to radiation and with radiation-induced DNA damage. However, contrarily to NF-κB canonical pathway inhibitors (like BAY 11-7082), that ultimately lead to reduced expression and nuclear translocation of NF-κB p65 subunit, there are fewer options to specifically inhibit the non-canonical NF-κB pathway, leading to RelB inhibition. Therefore, although NF-kB was found an interesting target, we were not able to fully reveal its role in macrophage radioresistance.

Effect of a single 2 Gy dose on macrophages

Before obtaining data to write the manuscript presented in this chapter, our study of the effect of ionizing radiation on human monocyte-derived macrophages was initiated with the characterization of their response to a single 2 Gy dose exposure. Macrophage signalling pathway activation and migratory profile upon exposure to 2 Gy were the main topics explored, as will be detailed herein.

Signalling pathway activation

To evaluate whether a single 2 Gy dose was activating signalling transduction in macrophages, the phosphorylation of some receptors known to be involved in response to radiation, particularly tyrosine kinase ones, was analysed.

i) Receptor tyrosine kinase phosphorylation

The phosphorylation of a wide range pannel of receptor tyrosine kinases (RTKs) was evaluated in 2 Gy irradiated macrophages, using a commercial RTK phospho-array, normalized to the basal phosphorylation levels of the same receptors in their non-irradiated counterparts (Figure C4).

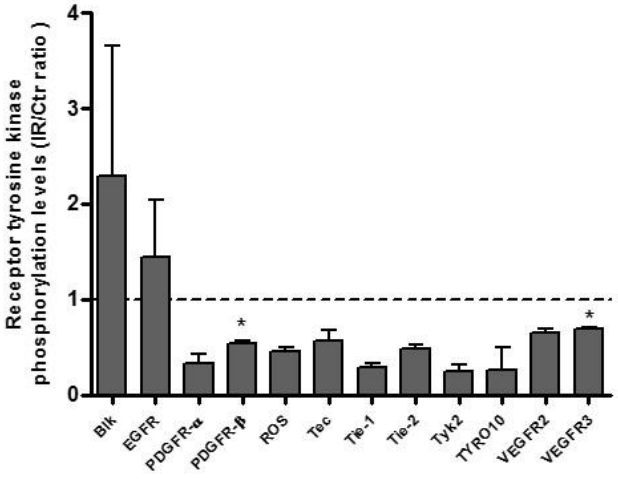


Figure C4 – A single 2 Gy doses decreases the phosphorylation of PDGFR-β and VEGFR3 in macrophages. About 1 h after macrophage exposure to a singe 2 Gy dose, protein extracts (n = 2) were collected and the relative level of tyrosine phosphorylation of a wide range of RTKs was evaluated, using a Human RTK Phosphorylation Antibody Array (RayBiotech). The signal was visualized through membrane exposure to X-ray films and signal intensities were quantified by densitometry using the QuantityOne software (BioRad). Graph represents the protein phosphorylation ratio of irradiated versus non-irradiated macrophage samples. Upregulation was considered if the ratio was ≥ 1.5 (upper dash line), while downregulation was considered if that ratio was <1.5 (lower dash line). * $P < 0.05$.

Results demonstrated that the phosphorylation of PDGFR (α and β), ROS, Tie (-1 and -2), Tyk2, TYRO10 and VEGFR2/3 receptors in macrophages tend to decrease upon irradiation, being the decrease in PDGFR-β and VEGFR3 statistically significant ($P < 0.05$). The phosphorylation level of

other receptors, like Blk and EGFR, appear to be altered in only one of the two blood donors tested. This interesting tendency of reduced RTK phosphorylation in irradiated macrophages may be explained by several factors, including a possible increased activity of protein phosphatases (PPs). For instance, PP1 activity, which is a major protein Ser/Thr phosphatase involved in several biological processes, was activated upon irradiation of Jurkat cells (human T cell lymphoma cell line)[1].

EGFR phosphorylation by irradiation – a validation step

Although EGFR was not consistently altered in both donors, we got particularly interested in this molecule because it is a tyrosine kinase receptor involved in distinct cellular processes, namely cancer cell invasion[2]. Despite some reports of EGFR expression on macrophages, like in melanoma[3], induced-colitis[4] and atherosclerotic plaques[5], its role on macrophages remains unclear. Therefore, we evaluated by immunocytochemistry analysis, whether macrophage EGFR phosphorylation was altered upon exposure to ionizing radiation (Figure C5).

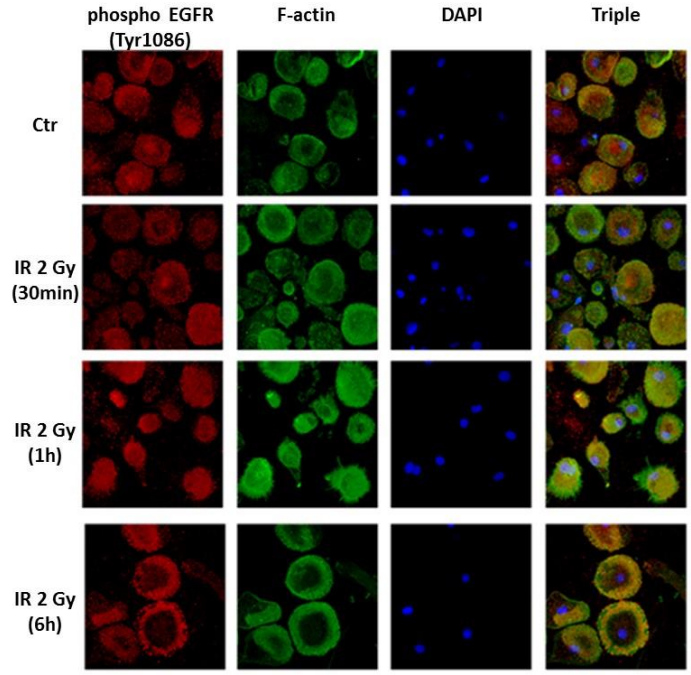


Figure C5 – A single 2 Gy dose induces EGFR tyrosine phosphorylation (Tyr1086) and recruits the receptor to cell periphery, in close proximity to macrophage podosomes. The tyrosine phosphorylation status of EGFR on Tyr1086 (*red*) residue and actin (*green*), was evaluated 30 min, 1 and 6 h after macrophage irradiation (2 Gy). Nuclei were counterstained with DAPI (*blue*). Pictures were obtained at 400x magnification with a high-resolution confocal microscope (Zeiss).

In non-irradiated macrophages, the endogenous levels of phosphorylated EGFR (Tyr1086) were reduced and homogenously distributed along the cytoplasm. After irradiation, the levels of phosphorylated receptor increased, becoming particularly concentrated at perinuclear areas. Notably, 6 h after irradiation, an intense EGFR phosphorylation was visualized at the cell periphery at areas of basal podosomes, without signs of colocalization.

ii) Phosphorylation of FAK, Src, AKT, p38 and ERK

These observations led us to investigate whether proteins downstream EGF receptor, commonly involved in radiation response, could also be activated by a single ionizing radiation dose. Therefore, the phosphorylation of FAK, c-Src, ERK, p38, and Akt was evaluated in irradiated macrophages, by western blot analysis (Figure C6). Results demonstrate that a single 2 Gy dose did not seem to alter FAK, p38 or ERK1/2 macrophage phosphorylation levels and possible alterations in the phosphorylation levels of c-Src and of Akt would have to be confirmed and validated in more donors.

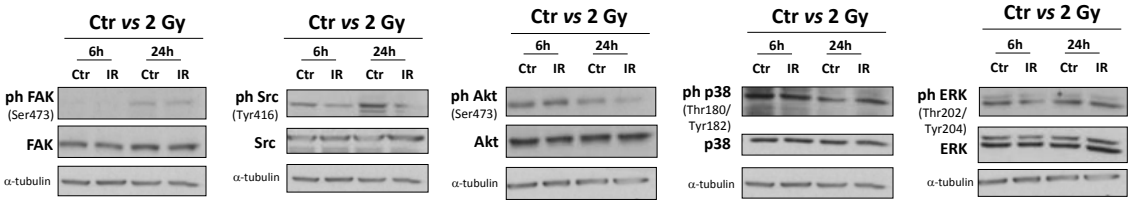


Figure C6 – A single 2 Gy dose is not inducing major alterations in the phosphorylation of FAK, p38 or ERK1/2 phosphorylation in macrophages. The phosphorylation status of FAK (Ser473), Src (Tyr416), Akt (Ser473), p38 (Thr180/Tyr182) and ERK1/2 (Thr202/Tyr204) was evaluated by western blot analysis at 6 and 24 h after macrophage exposure to a single 2 Gy dose.

Migration

Since migration is one of the main properties of macrophages and that EGFR activation is frequently related with enhanced cell migration and motility, we evaluated the impact of ionizing radiation on macrophage movement through time-lapse microscopy (Figure C7). We observed that while the migratory capacity of the majority of the population did not seem to be affected by irradiation, there was a subpopulation of macrophages that presented elongated cell morphology and long cellular protrusions, which clearly exhibited enhanced migration. Although no mechanisms potentially responsible for this effect were further investigated, we may consider the alterations of EGFR phosphorylation as an interesting candidate. Additionally, a more comprehensive analysis of the highly-migratory subpopulation would provide insights on the molecular mechanisms underlying this radiation-mediated macrophage migration.

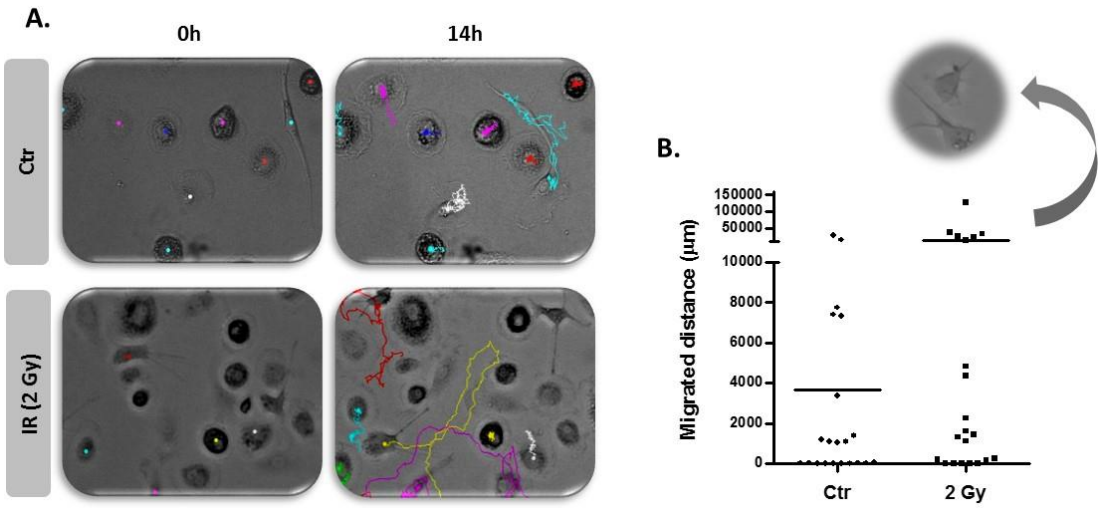


Figure C7 - Ionizing radiation seems to increase the migration of a small population of macrophages. Macrophages (*n* = 1) were plated on glass coverslips and exposed to a single 2 Gy dose. After stabilization in the incubator, coverslips with irradiated macrophages were transferred to the same plate of non-irradiated ones and cell movement was recorded every 5 min during 14 h, using a 20x objective and a DMIREZ time-lapse microscope (Leica). **A)** Images represent the time-lapse microscopy analysis of the trajectories of irradiated and non-irradiated macrophages at 0 and 14 h time-points. **B)** Quantification of migrated distance is represented in the graph. Cell trajectories were quantified with Fiji software considering 21 cells per condition, being each cell represented with a single point in the graph. The macrophage subpopulation exhibiting higher migratory capacity is shown in the picture above the graph.

Altogether, the results characterizing macrophage response to a single 2 Gy dose demonstrated that irradiated macrophages i) tend to decrease the phosphorylation of some RTKs, namely PDGFR (α and β), ROS, Tie (-1 and -2), Tyk2, TYRO10 and VEGFR2/3; ii) present a particular cellular distribution of phosphorylated EGFR (Tyr1086); and iii) seem to exhibit increased migration, but only in a small subpopulation. Although these preliminary results seem interesting, we aimed to find stronger differences between irradiated and non-irradiated macrophages, what allied to the fact that macrophages exhibit a more radioresistant profile, led us to further explore the effect of cumulative ionizing radiation doses up to 10 Gy, on macrophages, as described in our manuscript.

References

1. Guo, C.Y., Brautigan, D.L. & Larner, J.M. Ionizing radiation activates nuclear protein phosphatase-1 by ATM-dependent dephosphorylation. *The Journal of biological chemistry* **277**, 41756-41761 (2002).
2. Cardoso, A.P. *et al.* Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. *Oncogene* **33**, 2123-2133 (2014).
3. Scholes, A.G., Hagan, S., Hiscott, P., Damato, B.E. & Grierson, I. Overexpression of epidermal growth factor receptor restricted to macrophages in uveal melanoma. *Archives of ophthalmology* **119**, 373-377 (2001).
4. Lu, N. *et al.* Activation of the epidermal growth factor receptor in macrophages regulates cytokine production and experimental colitis. *Journal of immunology* **192**, 1013-1023 (2014).
5. Lamb, D.J., Modjtahedi, H., Plant, N.J. & Ferns, G.A. EGF mediates monocyte chemotaxis and macrophage proliferation and EGF receptor is expressed in atherosclerotic plaques. *Atherosclerosis* **176**, 21-26 (2004).

Chapter summary

Our main achievements (**Research Article 1**) indicate that cumulative ionizing radiation doses up to 10 Gy:

- induce macrophages DNA damage, confirmed through increased phosphorylation of H2AX and Chk2, without affecting their viability, metabolic activity or apoptotic pathways, as demonstrated by the absence of caspase-3 and -7 as well as PARP cleavage. Instead, radiation activates macrophages pro-survival Bcl-xL and NF-kB pathway, enhancing the expression and nuclear translocation of NF-kB RelB subunit and slightly increasing cRel expression;
- modulate macrophages towards a more pro-inflammatory profile, as indicated by the enhanced expression of *CD80*, CD86 and HLA-DR, while reduced the expression of *CD163* and *MRC1* as well as the release of IL-10, an anti-inflammatory cytokine; Additionally, ionizing radiation decreases *IL6* and *VCAN* expression.
- maintain macrophage plasticity, meaning that irradiated macrophages are still able to polarize towards a pro- or an anti- inflammatory phenotype, upon exogenous stimulation (with LPS/IFN-γ or M-CSF/IL-10). Particularly, irradiated macrophages exogenously directed towards a pro-inflammatory phenotype exhibited increased expression of pro-inflammatory HLA-DR marker, compared with non-irradiated counterparts. A significant decrease of IL-10 release is also observed in irradiated macrophages compared with non-irradiated ones, independently of the exogenous stimuli;
- do not affect macrophages proteolytic activity, although enhancing their morphology (area and aspect ratio) and phagocytic activity;
- maintain macrophage ability to promote cancer cell invasion and cancer cell-mediated angiogenesis.

Additionally (**complementary unpublished results**):

- irradiated macrophages exhibit decreased expression of some NF-κB targets at specific time-points, as of *IL1B*, *CCL2*, *CCL5* (1 h after 10 Gy) and *CXCL8* (6 h after 10 Gy);
- macrophages exposed to a single 2 Gy dose present: i) a reduction in the phosphorylation of some RTKs, ii) a particular cellular distribution of phosphorylated EGFR (Tyr1086) and iii) a tendency to increase migration, although only in a small subpopulation.

Chapter IV

Research Article 2

Proteomic signature of human macrophages exposed to clinical ionizing radiation doses

| Revised version resubmitted to *Journal of Proteome Research*

Chapter overview

After the characterization of macrophage response to radiation from a functional point of view (topic 1A) (Chapter III), the present chapter also addresses the first aim (but the topic 1B) of this PhD thesis - *To understand the effects of ionizing radiation exposure on human macrophages, from a signalling perspective*. Aiming to characterize the macrophage protein expression alterations induced by radiation exposure, macrophages irradiated with 5 cumulative ionizing radiation doses (2 Gy/fraction/day) were used as working models, and gel-based and gel-free proteomic techniques as methodological approaches. Bioinformatic analysis also allowed the identification of the main biological processes altered. Overall, this chapter aims to provide a proteomic signature of irradiated macrophages.

Experimental results are mainly compiled in an original research manuscript untitled "*Proteomic signature of human macrophages exposed to clinical ionizing radiation doses*" with the respective supplementary information, which revised version was resubmitted to *Journal of Proteome Research*.

Research Article 2

Proteomic signature of human macrophages exposed to clinical ionizing radiation doses

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Abstract

Despite technological advances in radiotherapy, therapy resistance is still a major challenge in cancer management. Tumour-associated host cells, like macrophages, contribute to tumour progression and influence treatment outcome. Fundamental research aiming to understand how ionizing radiation affects macrophage signalling pathways, particularly those involved in radiation response, is needed to improve radiotherapy efficacy. To address it, we exposed human monocyte-derived macrophages to 2 Gy/fraction/day, mimicking a cancer patient´s fractionation scheme. After 5 cumulative doses (10 Gy), total protein expression was analysed by complementary gel-based and gel-free proteomic approaches. Gene ontology enrichment analysis revealed associations with ATP biosynthesis and glucose 6-phosphate metabolic process. Accordingly, we demonstrated that ionizing radiation reduced macrophage total ATP cellular levels and slightly decreased their glucose uptake, without affecting their metabolic activity, despite radiation-induced DNA damage. Additionally, we identified and validated that ionizing radiation induces macrophage downregulation of cathepsin D, a lysosomal protease involved in antigen processing/presentation and apoptosis induction, and upregulation of transferrin receptor (CD71), an iron-binding protein. Overall, the present study reveals the proteomic signature of irradiated macrophages and contributes to the discovery of new molecular targets potentially involved in macrophage response to radiation, which modulation may enhance cancer cell sensitivity to radiotherapy, increasing treatment efficacy.

Keywords: Tumour microenvironment; Human monocyte-derived macrophages**;** Ionizing radiation**;** Proteomics**;** 2-DE**;** iTRAQ**;** Cathepsin D; Transferrin receptor (CD71)

Introduction

Radiotherapy is a widely used and highly cost effective anti-cancer treatment modality[1-2]. Although physics and technological evolution in the field have largely contributed to improve local control, it is still necessary to improve radiotherapy targeting, control disease progression and predict treatment outcome[3-4]. The key may rely on a better understanding of the effect of ionizing radiation on tumour-associated host cells, as they are crucial for disease progression and treatment outcome, and are also comprised within the irradiated region[5-7]. Cancer cell death induced by radiation exposure produces death-signals, leading to the recruitment of more immune cells, including monocytes which differentiate into macrophages at the injured site[8].

Macrophages are important components of innate immunity. During inflammation they are recruited into tissues, polarize into different subsets and exert either pro-inflammatory or anti-inflammatory functions to ultimately restore tissue homeostasis[9,10]. Due to their sophisticated phagocytic ability, they also play an important role in dying cell clearance[11]. In many tumours, macrophages constitute the major inflammatory stromal component and have been described as obligate partners for cancer cell migration, invasion and metastasis[12,13]. Additionally, macrophages are also involved in matrix deposition and remodelling, as well as response to hormones and chemotherapeutic agents, which makes them excellent targets to improve anti-cancer therapies[14,15].

Due to their role in tissue homeostasis and cancer progression, it is relevant to understand how macrophages respond to ionizing radiation exposure. Several studies have revealed that macrophages display a more radiation resistant phenotype than other immune cell populations, such as monocytes[16,17]. Additionally, our previous work demonstrated that irradiated macrophages remain viable and metabolically active, still promoting cancer cell invasion and cancer cell-induced angiogenesis, which is a major concern that needs to be addressed to improve radiotherapy efficacy[18].

Nevertheless, studies aiming to identify the molecular mechanisms possibly responsible for macrophage response to radiation are required. The majority of the investigations performed so far have used mouse models and single, low (< 0.1 Gy) or moderate (0.1 Gy – 1 Gy) ionizing radiation doses[19-23]. However, these doses are not relevant in a human clinical context, as radiotherapy is usually delivered in a multi-fractionated regimen, with daily doses of typically 2 Gy

(5 time per week)[24]. In summary, the model systems analysed as well as the dose of radiation frequently used still constitute clear limitations on clarifying the clinical effect of ionizing radiation on macrophages.

Recently, proteomic research tools, either on cells, tissues, or biofluids have gained a special interest in the radiation biology community, as they constitute broader and complex approaches, required for a detailed understanding of the signalling processes affected by ionizing radiation[25]. However, to the best of our knowledge, very few studies used proteomic tools to investigate the effect of ionizing radiation on macrophages. The first study dates from 1999, not resorting to mass spectrometry, and the others from 2005 and 2009 making use of mouse macrophages[26-28]. In summary, none of them explored the effect of clinically-relevant ionizing radiation doses on human macrophages. Therefore, in the present study, primary monocyte-derived macrophages were used as an *in vitro* model and exposed to cumulative X-ray fractions (2 Gy/fraction/day), mimicking a week of a cancer patient´s fractionation scheme. Protein expression changes in irradiated as compared to non-irradiated macrophages were then evaluated through two complementary methodological approaches: two-dimensional gel electrophoresis (2-DE) and labelling with isobaric tag for relative and absolute quantitation (iTRAQ) followed by 2D-LC-MS/MS, which will be designated from now on as gel-based and gel-free proteomic techniques, respectively. The discovery of ionizing radiation-induced protein expression alterations in human macrophages will help to better understand the mechanisms responsible for macrophage response to radiotherapy, modulation of which could increase cancer cell sensitivity to radiotherapy or decrease normal tissue reactions. Moreover, this knowledge could improve treatment outcome, through the combination of radiotherapy with other therapeutic strategies targeting macrophage-associated processes.

Results

In the present study, a combinatory approach between gel-based (2-DE) and gel-free (iTRAQ followed by 2D-LC) proteomic analysis was employed to understand the effect of cumulative and clinically relevant ionizing radiation doses (5 x 2 Gy) on human macrophage proteome (Figure 1). All experiments were conducted using monocyte-derived macrophages from distinct healthy blood donors, considered as biological replicates.

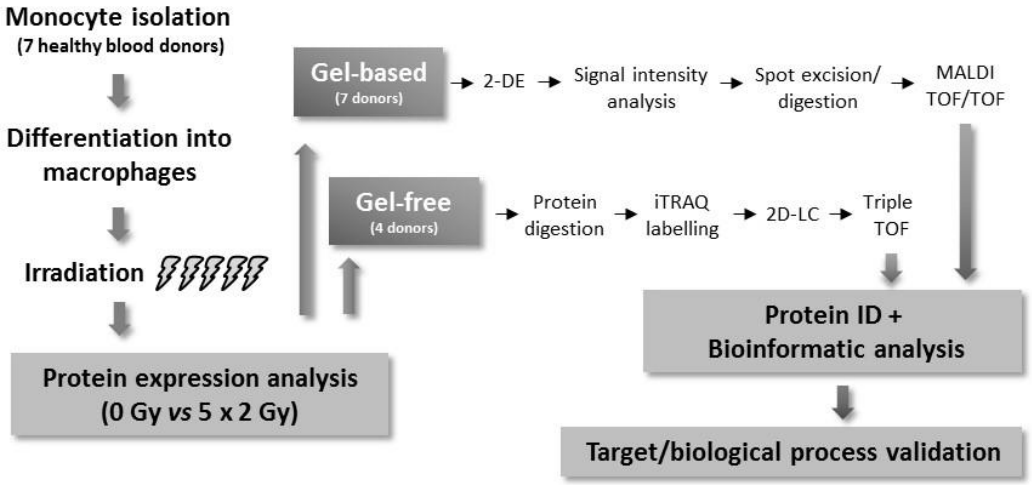


Figure 1 - Schematic representation of the methodological strategy performed in the present study. Briefly, buffy coats, obtained from 7 healthy blood donors, were used to isolate human monocytes. Monocytes were differentiated *in vitro* into macrophages, and further subjected to cumulative ionizing radiation doses, for 5 days (5 x 2 Gy). Control macrophages (0 Gy) remained as non-irradiated. Protein from irradiated and non-irradiated macrophages was extracted and protein expression analysis was developed through two proteomic approaches: gel-based (two-dimensional gel electrophoresis - 2-DE) and gel-free (iTRAQ followed by 2D-LC). In the gel-based approach, total macrophage (*n* = 7) protein extracts were loaded on 2-D gels and protein spot signal intensity was analysed. Differentially expressed proteins were excised, digested and identified by MALDI-TOF/TOF mass spectrometry. In the gel-free approach, total macrophage (*n* = 4 from previous *n* = 7) protein extracts were used. Proteins were digested, labelled, separated by two-dimensional liquid chromatography (2D-LC) and identified by Triple-TOF mass spectrometry. Data from both methodological approaches was analysed with bioinformatics tools and protein targets/biological processes were further validated.

Gel-based analysis

To evaluate proteins differentially expressed between irradiated (5 x 2 Gy) and non-irradiated macrophages, a comparative analysis using 2D-PAGE gel electrophoresis was first performed (Supplementary Figure S1). From a global protein spot comparison of the obtained gels, no major differences, in terms of protein spot number or density, were observed between the protein profile of irradiated and non-irradiated macrophages. This may suggest that ionizing radiation does not cause abrupt changes in macrophage proteome profile, at least in the dynamic-range covered by 2-DE gels and sensitivity detected with Coomassie staining. However, it does not necessarily mean that ionizing radiation does not induce some slight, but indeed significant, protein expression alterations in macrophages. In fact, a detailed analysis based on quantitative data (fold-change) and statistical tests (≥ 95 % confidence level) revealed that 17 protein spots were differentially expressed between irradiated and non-irradiated macrophages. According to the ratio (5 x 2 Gy/0 Gy) between experimental and control groups, 10 proteins were

downregulated in macrophages after ionizing radiation exposure, while 7 were upregulated. The identification of these protein spots is listed in Supplementary Table S1.

Considering the *identifier codes* (*IDs*) of proteins identified in the gel-based approach, an enrichment analysis regarding *biological processes*, *molecular functions* and *cellular components* of gene products was performed (Supplementary Figure S2). Regarding biological processes, the gene ontology (GO) analysis indicated that the differently expressed proteins were significantly associated with *regulation of biological quality*, which by definition refers to "any process that modulates a qualitative or quantitative trait of measurable attribute of an organism or part of an organism (such as size, mass, shape, colour, etc)"[40]. An association with metabolism, namely *catabolic processes* (like *organic substance catabolic process*) was also evidenced by this GO analysis. Amongst proteins associated with *response to stimulus*, the 60 kDa heat shock protein (mitochondrial) (*HSPD1*) and the peroxiredoxin-1 (*PRDX1*) were particularly associated with *response to stress*. Regarding their molecular functions, most of the identified proteins presented predicted *catalytic activity*, like aconitase 2 (mitochondrial) (*ACO2*) and cathepsin D (*CTSD*), including *oxireductase activity*, as was the case of alcohol dehydrogenase [NADP(+)] (*AKR1A1*). Finally, the GO analysis indicated that the majority of the identified proteins were mainly located in the cytoplasm, either in *mitochondria, cytosol* or even associated with *cytoplasmic vesicles* (as it was the case of cathepsin D).

Additionally, protein-protein interaction analysis, obtained through ClueGo plugin from Cytoscape (Supplementary Figure S3), indicated that the identified proteins were negatively associated (nodes with red dark colour) with the *regulation of platelet aggregation*, *neurotransmitter biosynthetic process* and *"de novo" protein folding*, evidenced by *HSPD1*, while positively associated (nodes with green dark colour) with *aerobic respiration*, *regulation of transcription factor import into nucleus namely NF-κB,* supported for instance by *PRDX1*, and *ATP biosynthetic process*.

Gel-free analysis

In parallel, iTRAQ labelling followed by 2D-LC-MS/MS was also performed as a complementary approach to evaluate macrophage protein expression alterations, after exposure to cumulative ionizing radiation doses (5 x 2 Gy). A total of 1343 protein groups were identified with 95% confidence level (Supplementary Table S2 and Supplementary Table S3), but only 1117, corresponding to those with at least 2 peptides used for quantification (Supplementary Table S4 and Supplementary Table S5), were considered for comparative analysis between irradiated and

non-irradiated macrophages. In the end, four expression ratios per protein, corresponding to the different donors used in the gel-free approach, were obtained. Only proteins presenting a *P* value < 0.05 in one-sample *t*-test and a fold-change ≥ 1.3 (upregulated proteins) or ≤ 0.77 (downregulated proteins) in at least 3 donors, were considered differentially expressed between experimental and control conditions (Supplementary Table S6). From the 67 proteins corresponding to these criteria, 60 of them were downregulated and 7 were upregulated in irradiated macrophages.

The distribution ratio of these 67 proteins, per blood donor, evidenced the emergence of three main nodes, as indicated by a heat map (Figure 2). According to a GO analysis, the first node was enriched in biological processes associated with *protein localization*, *vesicle-mediated transport*, *cellular aldehyde metabolic process* and *glucose 6-phosphate metabolic process*, while the second node was associated with *positive regulation of viral release from host cell*, *heterocycle catabolic process* and *mRNA catabolic process*. Finally, the last node was associated with *protein complex subunit organization* and *inorganic transmembrane transport*. Regarding cellular component and KEGG pathways, the GO analysis also demonstrated that all these 67 proteins were associated with membrane-bound vesicles, as also indicated by gel-based GO analysis, and with phagosome signalling pathways.

Additionally, the variability of profile of these 67 protein expression ratios between distinct donors reflected the individual response to ionizing radiation exposure. Accordingly, a cluster analysis of the protein expression profile of irradiated macrophages from the 4 donors evaluated revealed the existence of a main cluster including *donors D*, *G* and *F*, being *donor D* closer to *donor G*, while *donor E* was located in a distinct cluster (Figure 2). Interestingly, both *donors D* and *G* presented two common characteristics, being both male individuals with A^+ blood type. Despite this curious observation, the reduced number of donors used in the gel-free approach did not allow further analysis of a possible association between donor characteristics (blood type, gender and age) and macrophage response to radiation.

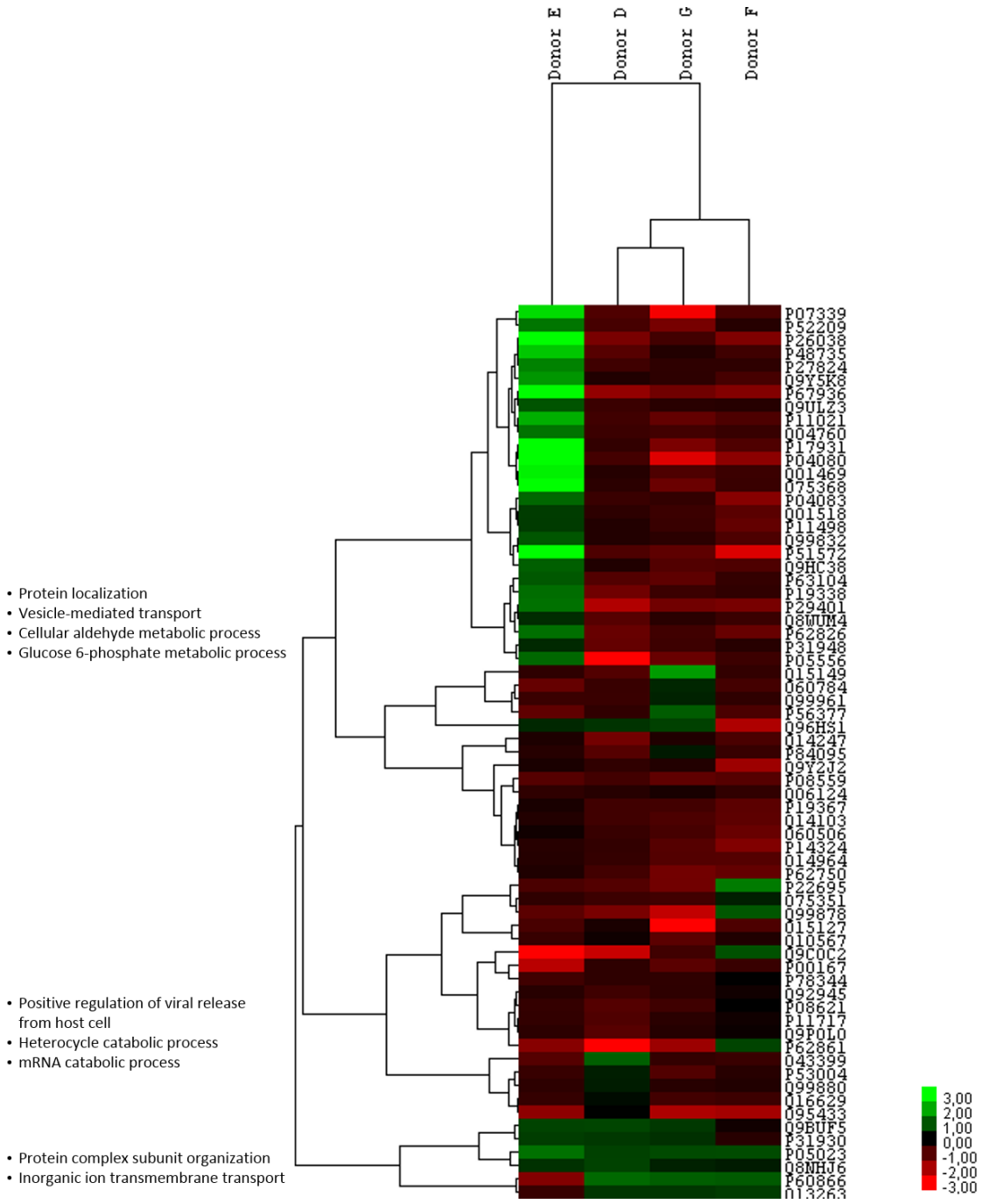


Figure 2 - Distribution ratio, per blood donor, of the 67 proteins differentially expressed in irradiated macrophages (5 x 2 Gy), in comparison with the non-irradiated ones, as obtained from gel-free analysis. Proteins found to be upregulated are annotated in green while downregulated ones are annotated in red. The biological processes indicated in each node were obtained through STRING.

To complement the GO enrichment analysis, protein-protein interactions were evaluated (Supplementary Figure S4), through ClueGo plugin from Cytoscape, evidencing an association with the following biological processes: *protein folding in endoplasmic reticulum*, *regulation of viral release from host cell* ($P < 0.0005$), *antigen processing and presentation of peptide antigen via*

MHC class II, *protein localization to endoplasmic reticulum*, *(positive regulation of) exosomal secretion*, *monosaccharide metabolic process*, *cellular aldehyde metabolic process* ($P < 0.005$), *post-Golgi vesicle-mediated transport, multivesicular body assembly*, *positive regulation of organelle assembly*, *virion assembly, spindle organization, negative regulation of cellular amide metabolic process, monosaccharide biosynthetic process*, *pyruvate metabolic process*, *glucose 6-phosphate metabolic process* and *aerobic respiration* ($P < 0.05$).

Protein interaction analysis (Supplementary Figure S5) also suggested that the majority of the identified biological processes were significantly downregulated, particularly *protein folding in endoplasmic reticulum* (mainly supported by vesicle-associated membrane protein-associated protein A (*VAPA*)), *antigen processing and presentation of peptide antigen via MHC class II* (supported by cathepsin D (*CTSD*)), *maintenance of protein location in cell* (supported by 78 kDa glucose-regulated protein (*HSPA5*) and hexokinase-1 (*HK1*)), *regulation of cell shape* (evidenced by moesin (*MSN*)), *cellular response to alcohol, negative regulation of cellular amide metabolic process, regulation of mRNA metabolic process* and *cellular aldehyde metabolic process*.

Validation of biological processes/targets

Despite DNA damage, irradiated macrophages remain metabolically viable, and reduce both glucose uptake and total cellular ATP levels

To confirm the induction of DNA damage by the selected fractionated irradiation protocol, the phosphorylation levels of histone H2AX (Ser139) (ɣH2AX), a sensitive marker of DNA-double strand breaks[41], was evaluated by western blot analysis in total lysates from irradiated (5 x 2 Gy) and non-irradiated macrophages (Figure 3A). Protein band quantification demonstrated that irradiated macrophages tend to present, in average, 2.5 times more phosphorylated H2AX than non-irradiated ones (Figure 3A). However, this did not necessarily indicate that ionizing radiation was causing macrophage cytotoxicity as in fact, irradiated macrophages presented similar metabolic activity as their non-irradiated counterparts (Figure 3B). Macrophage metabolic activity was determined through resazurin reduction, which is a redox dye commonly used as an indicator of cell cytotoxicity[37]. This is considered a simple and non-destructive assay to measure cell response to irradiation[42].

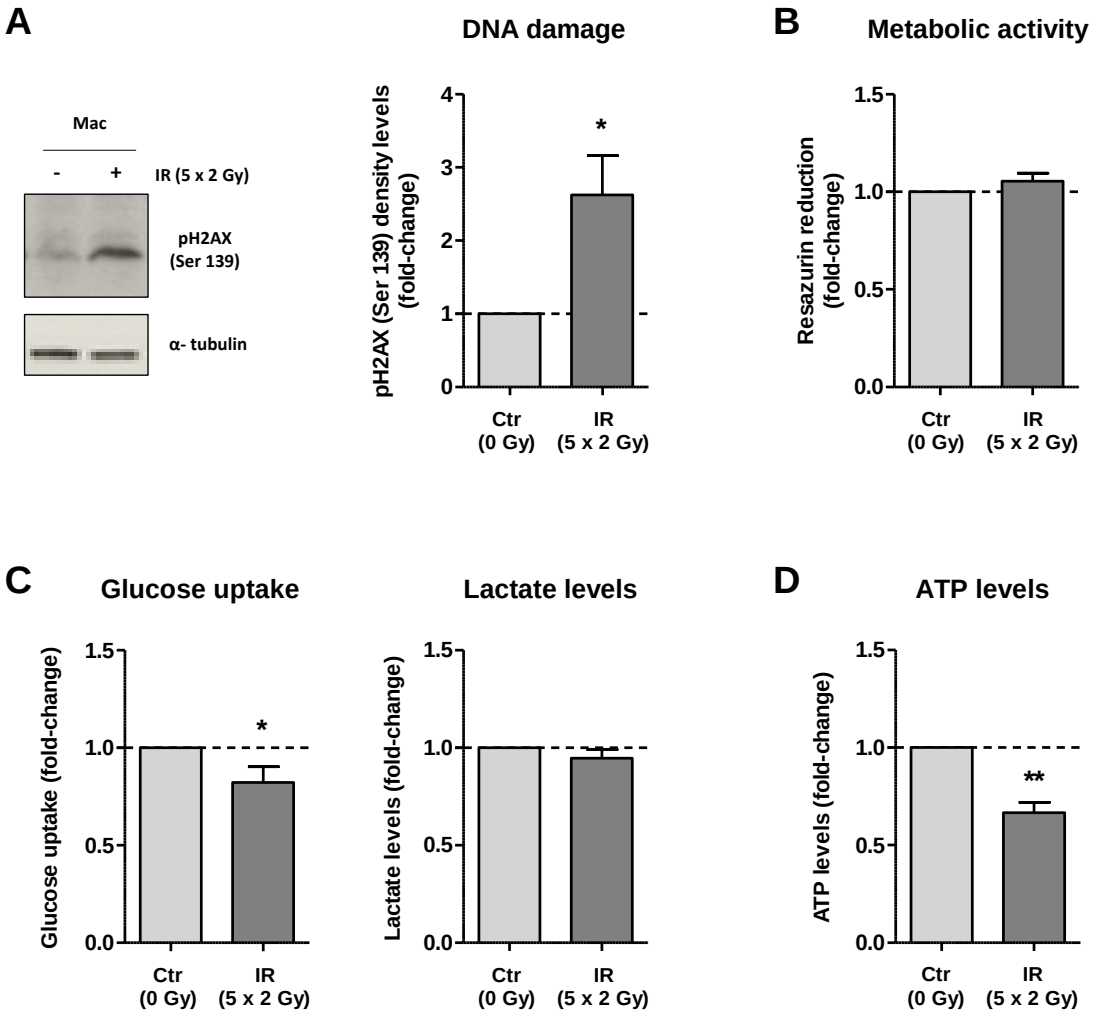


Figure 3 - Despite DNA damage, irradiated macrophages remain metabolically viable, and reduce both glucose uptake and total cellular ATP levels. A) DNA damage induced by cumulative ionizing radiation doses (5 x 2 Gy) in macrophages was confirmed by western blot analysis for H2AX phosphorylation (Ser139) (ɣH2AX), 40 min after radiation exposure. Quantification of ɣH2AX band intensity of irradiated macrophages, normalized to the control, is represented in the graph (*n* = 3). **B)** Metabolic activity of irradiated macrophages (*n* = 8) was measured through resazurin reduction assay and normalized to that of non-irradiated ones. **C)** Both glucose (*n* = 12) and lactate (*n* = 14) levels were determined in macrophage conditioned medium (CM) (*n* = 7). To obtain glucose uptake, glucose levels were subtracted to the initial glucose concentration of RPMI medium. **D)** ATP was measured after macrophage lysis (*n* = 6).

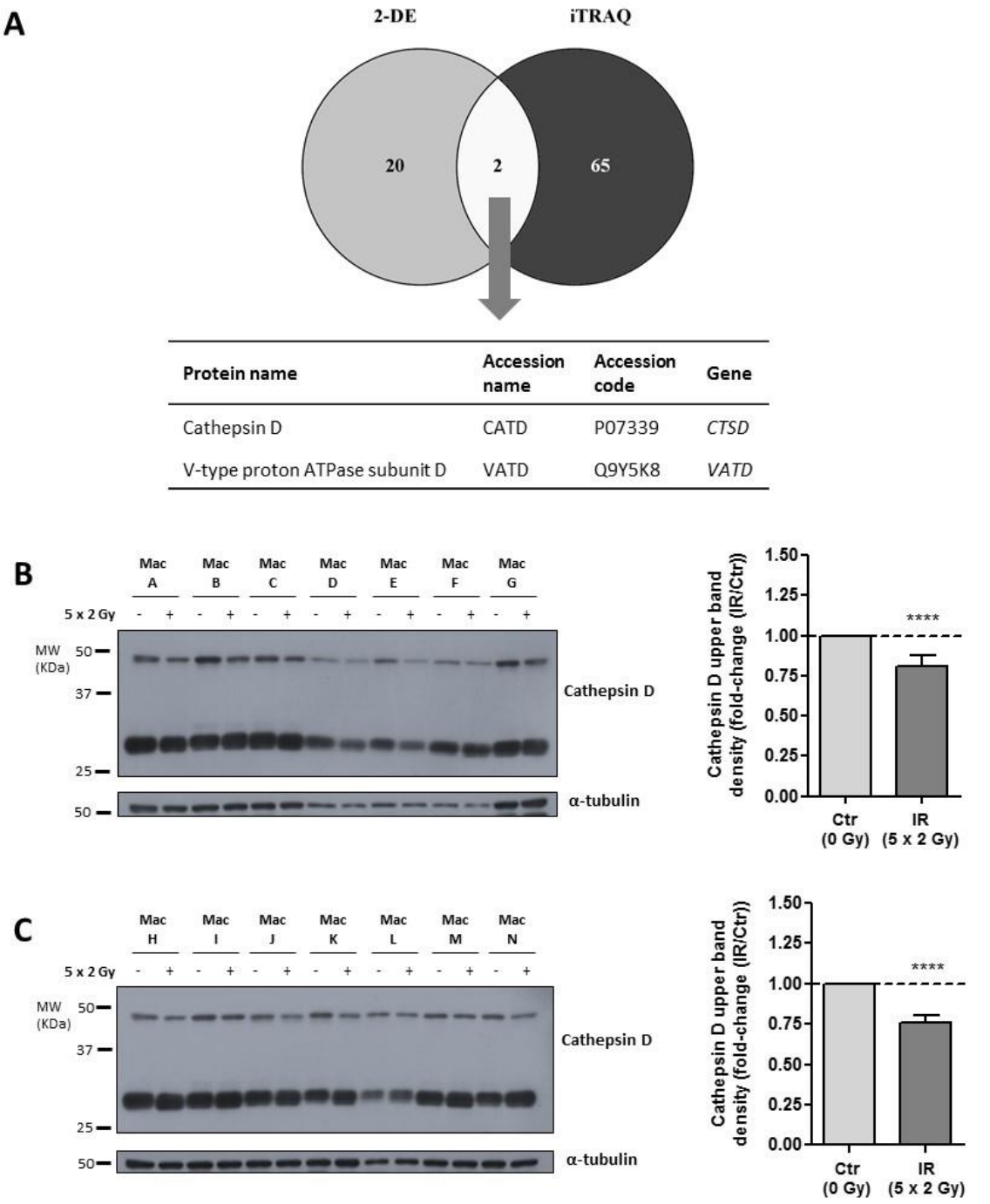
Additionally, as targets obtained from iTRAQ were associated with *glucose 6-phosphate metabolic process*, the glucose uptake was evaluated and complemented with the determination of lactate levels in irradiated and non-irradiated macrophages (Figure 3C). Results demonstrated a significant reduction of glucose uptake, without alterations in lactate levels. Finally, since the gel-based approach suggested a deregulation of *ATP biosynthetic process*, the effect of ionizing radiation on total cellular ATP levels was also evaluated, evidencing a significant reduction in irradiated macrophages (Figure 3D). It is important to be aware that total ATP levels result from

the balance between cellular ATP production (mainly from complete glucose oxidation) and ATP consumption through major ATP consumers (like protein synthesis and Na^+/K^+ ATPases, which transports Na^+ and K^+ ions across the plasma membrane in exchange of ATP molecule split[43-46]). Thus, a reduction of total cellular ATP levels in irradiated macrophages may be justified by the combination of i) a decreased glucose uptake, which probably results in a reduced glucose oxidation, and ii) the upregulation of the Na^+/K^+ ATPase alpha 1 subunit (*ATP1A1*), an isoform of the Na^+/K^+ ATPase catalytic subunit, as suggested by iTRAQ data.

Cathepsin D is downregulated in irradiated macrophages

Through conjugation of protein IDs from targets identified in gel-based approach together with those from gel-free approach, 2 common targets were found: cathepsin D and V-type proton ATPase subunit D (Figure 4A), which expression was further evaluated by western blot analysis.

Studies on cathepsin D biosynthesis revealed that it is first synthetized in the rough endoplasmic reticulum as preprocathepsin D (54 KDa), cleaved into procathepsin D (52 KDa), and then subjected to post-translational modification events and transported to the Golgi apparatus, where it acquires the mannose-6-phosphate recognition signal for endosomal/lysosomal transport[47-50]. After being converted into the active one-chain form (48 KDa) in lysosomes, cathepsin D is finally cleaved, although exhibiting the same proteolytic activity, into two chains, a heavy (34 KDa) and a light one[48]. In the present work, western blot analysis for cathepsin D led to the identification of two bands, one below 50 KDa, probably corresponding to the active chain form, and the other between 25 and 37 KDa, suggested as the mature form. Evaluation of cathepsin D expression in macrophage protein extracts, from the 7 donors used for both gel-based and gel-free approaches, confirmed that the active chain form was downregulated in irradiated macrophages, when compared to non-irradiated ones (Figure 4B). This observation was extended to macrophage protein extracts from 7 additional donors (Figure 4C). Although downregulation of cathepsin D was successfully validated, the same was not applied to V-type proton ATPase subunit D, as no major expression differences were observed between irradiated and non-irradiated macrophages (Supplementary Figure S6).



Protein name	Accession name	Accession code	Gene
Cathepsin D	CATD	P07339	*CTSD*
V-type proton ATPase subunit D	VATD	Q9Y5K8	*VATD*

Figure 4 - Validation of cathepsin D downregulation in irradiated macrophages (5 x 2 Gy), compared with non-irradiated ones. A) Identification of IDs, through Venny software, of differentially expressed proteins, obtained from gel-based and gel-free approaches, in irradiated macrophages. **B)** Validation of cathepsin D downregulation, by western blot analysis, in the same donors (n = 7) used for gel-based and gel-free approaches and in **C)** additional donors (n = 7). Graphs represents the quantification of cathepsin D upper band intensity, normalized to α-tubulin staining, in irradiated macrophages and compared to that of non-irradiated macrophages. A one sample t-test was used for statistical analysis. **** $P < 0.0001$.

***Regulation of apoptosis* and *transferrin receptor transport*: two biological pathways suggested to be altered in irradiated macrophages**

After evaluating the protein expression of the two targets altered in irradiated macrophages, obtained through gel-based and gel-free approaches, we explored the biological processes in which they were involved as well as their correlation with expression alterations found in other targets. In order to address these issues, a mathematical network, based on complex algorithms, was built. It focused essentially on the relations between numbers, i.e., the 4 ratios for each protein, aiming to evidence potential functional relations between proteins, which may not yet be described from a biological point of view, or support already described ones. To build this network, the IDs and quantitative data from all 67 differentially expressed proteins, obtained from gel-free approach were used as inputs (see Supplementary Table S6). A protein increase upon macrophage irradiation (fold-change >= 1.3) was indicated by "acession code_I", while protein decrease (fold-change <= 0.77) was indicated by "acession code_D". From this new mathematical network, 10 communities, indicated by different colours, emerged (Supplementary Figure S7A). Particularly, the communities where the two main downregulated targets - cathepsin D and V-type proton ATPase subunit D were located are indicated by orange (Supplementary Figure S7B) and red (Supplementary Figure S7C) colours, respectively. The orange network evidences a closer relation between the downregulation of cathepsin D (CATD_D) and of two other proteins, adenylyl cyclase-associated protein 1 (CAP1_D) and annexin A1 (ANXA1 _D). Also related with downregulated cathepsin D were downregulated pyruvate carboxylase (PYC_D), tropomyosin alpha-4 chain (TPM4_D), 14-3-3 protein zeta/delta (1433Z_D), 78 kDa glucose-regulated protein (GRP78_D), plectin (PLEC_D) and moesin (MOES_D). Although we were not able to validate V-type proton ATPase subunit D (VATD_D) downregulation (Supplementary Figure S6), the green network indicates that this protein was closely associated with the downregulation of nucleolin (NUCL_D) and isocitrate dehydrogenase [NADP] (IDHP_D), and indirectly with fatty acid-binding protein (FABP5_D), galectin-3 (LEG_D) and hexokinase-1 (HXK1_D).

To investigate the biological relevance of such direct/indirect protein interactions, the IDs of all elements of orange or green colour communities were inserted into STRING, allowing a maximum of five additional protein interactors per each network. Cathepsin D and the other elements of orange community were significantly associated with *the apoptotic signalling pathway*, while V-type proton ATPase subunit D and its counterparts of the green community were associated with *transferrin receptor transport* and *phagosome maturation*. In order to validate alterations in *transferrin receptor transport*, we evaluated the expression of transferrin receptor protein 1 (TfR1,

also known as CD71), an iron binding transport protein, which we found to be upregulated in irradiated macrophages (Figure 5).

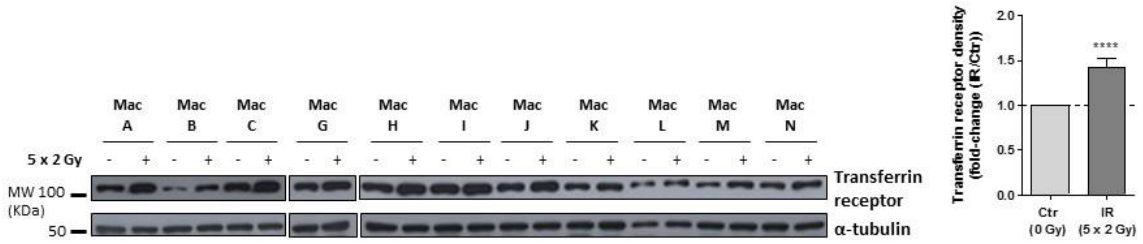


Figure 5 - Transferrin receptor is upregulated in irradiated (5 x 2 Gy) macrophages. Transferrin receptor expression was evaluated by western blot analysis. A combination of three of the donors used for gel-based and gel-free approaches (Mac A, B, C) together with eight additional donors (Mac G, H, I, J, K, L, M and N) was used. Graph represents the quantification of transferrin receptor band intensity, normalized to α-tubulin staining, in irradiated macrophages and compared to that of non-irradiated macrophages. A one sample *t*-test was used for statistical analysis. **** $P < 0.0001$.

Discussion

In the present study, we aimed to identify a profile, in terms of biological processes and protein expression, characteristic of macrophages exposed to clinically relevant fractionated ionizing radiation doses. In order to mimic one week of a cancer patients´ treatment, macrophages were irradiated with 2 Gy/fraction/day during 5 days, rather than with a single dose. As expected, we verified very high levels of DNA damage in macrophages 40 min after radiation exposure. Accordingly, in other models, almost every cells presented ɣ-H2AX foci 30 min immediately after radiation exposure, but this number was dramatically reduced to 33% 8 h later and at 24 h only 20% of the cells were still ɣ-H2AX foci-positive, similarly to non-irradiated cells[51]. This DNA damage repair kinetics led us to perform this proteomic study at a later time-point (24 h) after irradiation in order to evaluate other radiation-induced effects, besides intense DNA damage.

Investigating the direct effect of radiation exposure on macrophages is of major interest as, comparing to other inflammatory cells, they have been considered relatively radioresistant, and may contribute to tumour resistance to radiotherapy[16,17]. According to the literature, only two studies investigated the response of macrophages to ionizing radiation exposure using mass spectrometry-based proteomics. However, neither fractionated doses nor human macrophages were used. In 2005, Chen and colleagues demonstrated, through 2-DE Difference Gel Electrophoresis (DIGE), increased expression of actin cytoplasmic 1 expression in mouse macrophages when animals were exposed to a single whole-body dose of 0.5 Gy[27]. Expression

alterations in additional targets were found to be mouse strain-dependent. In 2009, Smallwood and colleagues identified an ionizing radiation dose-dependent increase in the expression of the calcium regulatory protein calmodulin (CaM) in mouse macrophages (RAW 264.7) upon irradiation[28]. Additionally, a mass spectrometry approach, using isotopic labelling, evidenced minor differences in other proteins in macrophages exposed to a single 1 Gy dose, indicating that CaM levels may be part of a radiation-dependent cellular response. In fact, CaM overexpression was suggested to increase DNA repair pathways, enhancing macrophage radioresistance. Although both studies provided important data, it is difficult to speculate whether a similar response would be observed in human macrophages, as mouse and human macrophages present many distinct features[52].

Recognizing the limitations, but most importantly the advantages of gel-based and gel-free proteomic approaches, we combined both strategies to cover a higher proportion of the proteome and to obtain complementary and a more complete set of data about the effect of ionizing radiation exposure on human macrophages, rather than to compare both methodologies. One of our main findings was the downregulation of cathepsin D in irradiated macrophages. Cathepsin D is one of the most abundant lysosomal proteases also present in endosomes and phagosomes, being the last structures responsible for the engulfment of bacteria and other particles[48,53,54]. Based on its ability to cleave a wide range of target substrates, cathepsin D has been involved in numerous physiological functions, namely protein degradation in the acidic milieu of lysosomes, antigen processing and also regulation of programmed cell death[55]. Accordingly, cathepsin D downregulation and reduced hydrolytic and serine proteolytic activities, were also found in phagosomes of LPS/IFN-ɣ stimulated macrophages[56,57]. These macrophages are generally designated pro-inflammatory and typically exhibit high antimicrobial capacity[58]. Consistently, cathepsin D upregulation and increased proteolytic activity were described in IL-4 stimulated macrophages, which display an anti-inflammatory phenotype[59]. This suggests that cathepsin D downregulation may correlate with increased phagocytic rate and reduced anti-inflammatory phenotype, both characteristics that we have previously found in irradiated macrophages[18]. Phagosomes of pro-inflammatory macrophages exhibited reduced proton-pumping activity when compared to those from anti-inflammatory ones, which difficult phagosome acidification[60]. Reduced acidification and proteolysis in phagosomes is indicative of a delayed fusion with lysosomes and consequent later maturation of phagolysosomes, which has been suggested as a facilitator of *antigen processing and presentation*, a biological process that we found to be downregulated in irradiated macrophages[56,57, 61].

Additionally, cathepsin D may also be involved in cell death signalling, as the release and diffusion of cathepsins and other hydrolases from the lysosomal lumen to the cytosol, caused by lysosome membrane permeabilization (LMP), leads to the degradation of vital proteins and causes damage to other cellular components, which is a mechanism of cell death induction[62]. Subsequently, cathepsin D can activate pro-apoptotic Bid, through specific cleavage sites[63], and cleaved Bid activates the intrinsic apoptotic pathway by binding to Bax, which leads to *mitochondrial outer membrane permeabilization* (MOMP) and consequent cytochrome c release[64]. Accordingly, microinjection of cathepsin D into the cytosol, which mimics LMP, caused cytochrome c release, activated caspases, and induced cell shrinkage, chromatin condensation and formation of pycnotic nuclei, which all together are indicative of apoptosis induction[65]. Cathepsin D activation triggered apoptosis also during pneumococcal infection in macrophages, while cathepsin D pharmacological inhibition blocked it[66]. Other molecules associated with apoptosis signalling were also suggested to be downregulated in irradiated macrophages, such as cytochrome b5, another identified substrate of cathepsin D that forms complexes with cytochrome c from mitochondria, an interaction supposed to play a role in apoptosis initiation[67,68], and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)), an adaptor molecule found to be a key mediator in apoptosis and inflammasome activation[69,70]. Altogether, the downregulation of several cell death-related proteins in irradiated macrophages is in agreement with our previous experimental data, which evidenced that despite DNA damage, irradiated macrophages remained viable and metabolically active[18]. Contrarily to irradiated macrophages, breast cancer cells exhibited increased cathepsin D expression upon exposure to single (10 Gy) or fractionated (5 x 2 Gy) radiotherapy, at the same time they highly reduced their viability[71], suggesting a relation between cathepsin levels and radiation-induced apoptosis. Our previous work demonstrated that ionizing radiation did not induce activation of pro-apoptotic proteins, like caspase-3/-7 or PARP, in macrophages nor even apoptosis-associated morphological features were observed[18]. Consistently, irradiated macrophages presented an increase of both NF-κB transcriptional activation (particularly of RelB subunit) and Bcl-xL expression, evidencing the promotion of pro-survival activity[18] (Figure 6 – *right side*). Accordingly, proteins identified through gel-based approach were positively associated with the *regulation of transcription factor import into nucleus, namely NF-κB* (Supplementary Figure S3). Overall, our findings corroborate previous descriptions of a macrophage radiation resistant phenotype[16,28,72], suggesting other possibly involved targets. We indeed hypothesize that RelB induction upon macrophage irradiation could somehow be related with cathepsin D downregulation and that both effects could lead to

increased macrophage survival after irradiation. Accordingly, cathepsin D downregulation was observed in breast cancer cells upon RelB overexpression[73].

Macrophages are important players in iron homeostasis, due to their ability to recycle iron. Particularly, macrophage engulfment of aged erythrocytes leads to their digestion inside phagolysosomes, liberating haemoglobin that contains a heme group, which after oxidation releases iron[74]. Another macrophage iron uptake process is through transferrin, an iron-binding protein[74]. After receptor-mediated endocytosis, iron dissociates from transferrin receptor within acidified endosome. Notably, the present proteomic study demonstrated that irradiated macrophages present increased expression of transferrin receptor protein 1 (TfR1, also known as CD71). In macrophages, upregulation of transferrin receptor frequently occurs during bacterial infection, being crucial for the proliferation of some intracellular pathogens[75]. Increased transferrin receptor expression also seems to be characteristic of M2 (anti-inflammatory) polarized macrophages, although its role remains to be clarified[76]. This, together with the possible involvement of cathepsin D downregulation in M1 (pro-inflammatory) macrophages and our previous data[18], could support the hypothesis that irradiated macrophages may exhibit features from both pro- and anti-inflammatory macrophages. Additionally, transferrin receptor was found to interact with IKK (IκB kinase), a protein complex involved in NF-κB activation, being required for its activity[77]. Regarding the effect of irradiation on the levels of transferrin receptor, it was reported that some splenic mononuclear cell populations exhibited upregulation of transferrin receptors following mice whole-body irradiation[78]. In human cells, higher transferrin receptor expression was found in far-ultraviolet (UV) light resistant cells rather than in UV-sensitive ones[79]. Accordingly, depletion of transferrin receptor reduced UV-resistance, while overexpression increased it, which was suggested to be associated with a possible anti-apoptotic effect of this growth factor[79]. Overall, we speculate that in irradiated macrophages, both cathepsin D downregulation and transferrin receptor upregulation, may be associated with apoptosis suppression and radiation resistance, which could be linked to the previously observed NF-κB RelB subunit activation[18].

In summary, we presented, for the first time, a global view of the biological processes and a proteomic signature for human macrophages exposed to fractionated ionizing radiation doses, mimicking one week of cancer patients´ treatment. This proteomic study involved gel-based and gel-free proteomic complementary methodologies revealing the downregulation of cathepsin D, a protein involved in cell death and antigen processing/presentation, and the upregulation of

transferrin receptor, which is involved in iron uptake. However, the possible role of these two targets in macrophage radioresistance phenotype requires further investigations. Our study also evidenced that irradiation may induce alterations in several macrophage biological processes, namely *post-Golgi vesicle-mediated transport*, *protein folding in endoplasmic reticulum* and *endosome organization*. In fact, as transferrin receptor and mature cathepsin D are considered early-endosomal and lysosome/phagolysosome markers, respectively, their deregulated expression in irradiated macrophages may suggest functional alterations in these cellular compartments[80], which should be further investigated. Similarly to other proteomic studies, this work constitutes an initial and broad strategy, which may provide important clues for further and detailed studies rather than definitive answers[81]. Nevertheless, it increases the general comprehension of macrophage radiobiology, providing new insights into the field. Additionally, further clarification of macrophage radiation resistant mechanisms may contribute to improve radiotherapy efficacy, either by increasing cancer cell death or by controlling normal tissue reactions to radiotherapy.

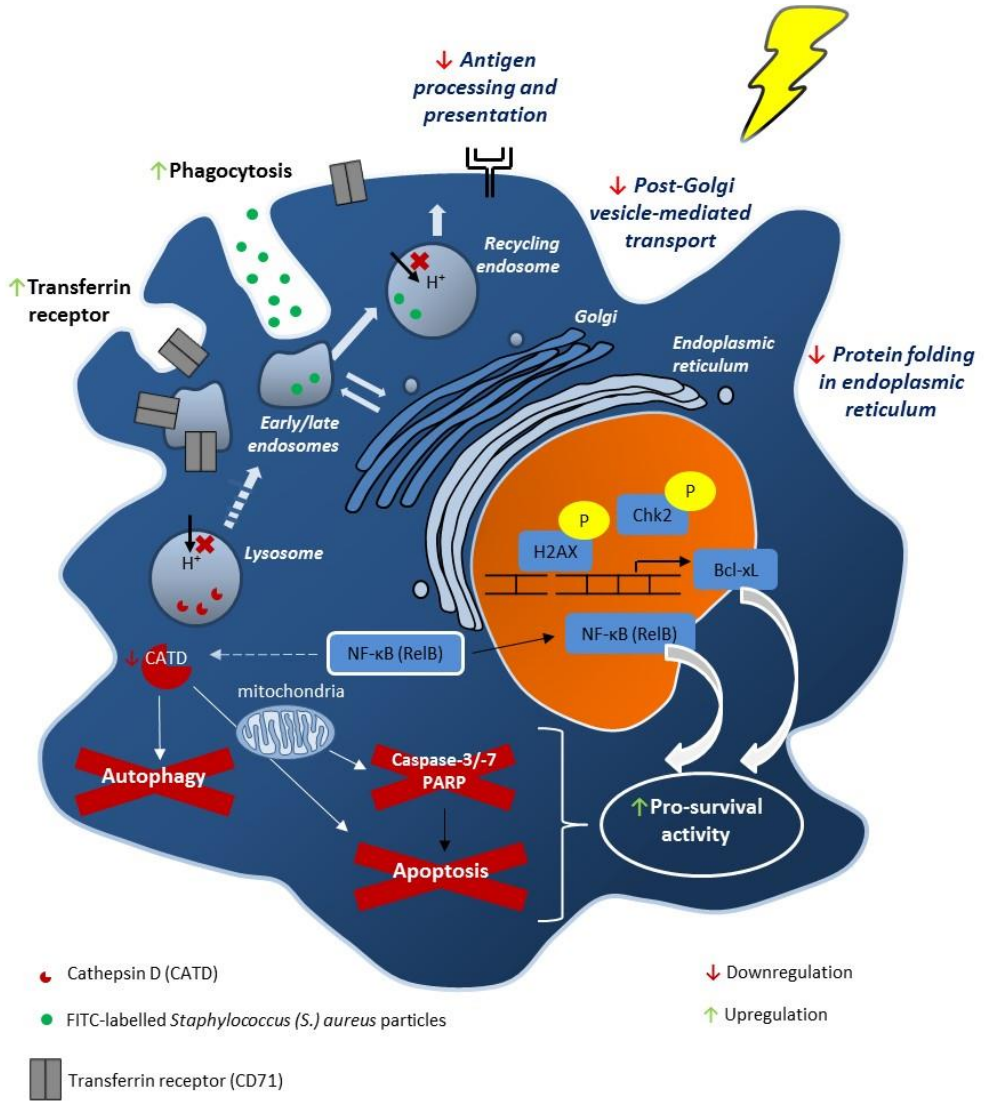


Figure 6 - A proposed model for fractionated ionizing radiation (5 x 2 Gy)-induced effects in human macrophages. This scheme was based on a literature review, as well as on findings obtained from the present proteomic study (*on the left*), and results from our previous work (*on the right*), which characterized the effect of ionization radiation on macrophages from a functional and signalling activation point of view. In summary, we hypothesize that cathepsin D downregulation in irradiated macrophages may contribute to increased phagocytosis, impaired antigen processing and presentation, and more importantly to cell death blockage. Together with increased Bcl-xL expression and RelB nuclear translocation, which we previously described in irradiated macrophages, cathepsin D downregulation could be involved in macrophage survival upon irradiation. According to the literature, an association may indeed exist between RelB overexpression and cathepsin D reduction. Additionally, transferrin receptor, which is involved in iron uptake, was found to be upregulated in irradiated macrophages. Altogether, expression alterations in transferrin receptor and cathepsin D, markers of early-endosomal and lysosome/phagolysosome markers, respectively, may suggest deregulation of these intracellular compartments in irradiated macrophages. In fact, iTRAQ data suggests alterations in several biological processes associated with endosomal compartments, such as *post-Golgi vesicle-mediated transport* and *protein folding in endoplasmic reticulum*.

Material and methods

Ethics statement

In the present study, human monocytes were obtained from buffy coats, which are a highly leukocyte-enriched waste-product that results from a whole blood donation, from healthy blood donors. A collaboration protocol between our Institution and Centro Hospitalar São João (CHSJ), where blood donations of Portugal North region are performed, allows the use of these products for investigation purposes. All studies using this human material were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. Informed consent was obtained from all subjects before blood donation.

Blood donor characteristics

The 7 anonymous blood donors involved in the proteomic approaches performed in the present study are Caucasian healthy individuals, presenting O^+ or A^+ ABO and Rh blood types, being 3 females and 4 males with ages between 26 and 55 years. Of note, the gel-based approach was performed with macrophage protein lysates from 7 blood donors, obtained in 4 independent irradiation experiments. However, due to technical limitations, the gel-free approach was only performed with macrophage protein lysates from 4 of the previous 7 blood donors, obtained in 2 independent irradiation experiments, being 2 female and 2 male donors randomly selected. Several additional anonymous donors were used for validation purposes.

Human monocyte isolation and macrophage differentiation

Human monocytes were isolated from healthy blood donors as previously described[29]. Following this procedure, over 80% of isolated monocytes were found to be CD14-positive[29]. For monocyte-macrophage differentiation, $1.2x10^6$ cells/9.6 cm^2 were cultured in RPMI1640 medium (with GlutaMax) (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), in the presence of 50 ng/mL of macrophage colony-stimulating factor (M-CSF) (ImmunoTools, Friesoythe, Germany). Culture medium was replaced after one week and macrophage differentiation was completed 13 days after monocyte isolation, as at this stage macrophages were shown to provide a higher stimulus for cancer-cell invasion, than with shorter differentiation times[29].

Ionizing radiation exposure

Prior to irradiation, a dosimetry plan was established (ELEKTA CMS XiO v.4.7.0). Culture plates were submitted to a Computerized Tomography (CT) scan and the volume occupied by two entire plates was defined as the target volume. Two beam fields, one anterior-posterior and other posterior-anterior, were arranged to deliver 2 Gy per fraction to this target volume. Inside the defined volume, the total dose varied from 198 cGy to 202 cGy. As the 4 cGy difference was not significant, the same dose was considered homogenously distributed through the plates. To guarantee this uniform dose and to avoid the build-up region of the 18 MV photon beam, 5 water plates were added above, and 5 below the culture plates during irradiation. Medium was renewed before the first irradiation. Macrophages were exposed to 5 cumulative ionizing radiation doses (2Gy/fraction/day), for 5 days (5 x 2 Gy), totalizing 10 Gy. Photon beam was produced by a PRIMUS (Siemens, Malvern, PA, USA) linear particle accelerator, used for human radiotherapy sessions, operated at 18 MV at the Radiotherapy Service of CHSJ. To avoid differences between non-irradiated and irradiated cells, caused by medium agitation during transport to/from the Radiotherapy Service, control cells were also transported, but were not radiation-exposed.

Protein extraction

To perform gel-based and gel-free approaches, protein from irradiated and non-irradiated macrophages from the same blood donor, was extracted about 24 h after ionizing radiation exposure (5 x 2 Gy). Lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% Igepal] was supplemented with a cocktail of proteases and phosphatases inhibitors: phenylmethanesulfonylfluoride 1 mM, sodium metavanadate 3 mM, sodium fluoride 20 mM, sodium pyrophosphate tetrabasic 25 mM (Applichem), aprotinin 10 mg/ml and leupeptin 10 mg/ml (Sigma-Aldrich). Protein concentration was determined with Protein Assay Dye Reagent Concentrate (BioRad).

Gel-based

Two-dimensional gel electrophoresis - 2-DE

After extraction, protein (n = 7) was precipitated according to manufacturer´s instructions (ProteoExtract Protein Precipitation Kit, Calbiochem, EMD Millipore, Germany). Protein pellet was ressolubilized, for 1 h under gentle agitation, into 240 μL of DeStreak rehydration solution (GE Healthcare, UK). Protein content was quantified using the 2-D Quant Kit (GE Healthcare, UK), according to manufacturer´s instructions. For the first dimension - isoelectric focusing (IEF), 0.2%

ampholytes (BioRad) were added and 200 μg of protein were pipetted along a channel in an isoelectric focusing tray (BioRad), followed by overnight passive immobilized pH gradient (*IPG*) *strips* rehydration at room temperature. IPG Strips (Ready Strip™, BioRad) (pH 3–10 NL, 11 cm) were then placed in the PROTEAN IEF cell (BioRad). IEF was carried out at 20°C constant temperature and 50 μA/strip amperage. The following voltages and running times were used: start voltage of 250 V for 15 min (Step 1), rapid voltage ramping of 2 h from 250 V to 8000 V (Step 2) and a final focusing of 8000 V until the focusing totalize approximately 25000 Vh (Step 3).

After the first dimension, each strip was incubated with equilibration buffer [6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol and 0.002% bromophenol blue] supplemented with 20 mg/mL of dithiothreitol (DTT) (Sigma-Aldrich, MO) and followed by the addition of equilibration buffer with 25 mg/mL of iodoacetamide (Sigma-Aldrich), 10 min each. Precast polyacrylamide gels (IPG+1-well, Any kD, 11 cm, BioRad) were used for the second dimension (10 min at 10 mA followed by 50 min at 12.5 mA). Gels were stained with Page Blue (Thermo Scientific), which is a ready-to-use solution based on Coomassie Brilliant Blue G-250 dye, and scanned with a GS800 densitometer (BioRad).

Protein expression analysis

2-DE gels protein expression analysis, involving spot comparison/matching and data analysis, was performed with PDQuest software (version 8.01, BioRad). Image was first automatically filtered and smoothed to clarify the spots. The 7 blood donors used in the present study were considered biological replicates and therefore 7 gel images, corresponding to irradiated macrophages (experimental group) and another 7 corresponding to non-irradiated macrophages (control group), were analysed at the same time. The same number of spots per gel was considered for comparison purposes. Images were normalized using local regression method. Each spot intensity, given by optical density units, was determined. Comparison of differentially expressed protein spots between the two groups was first based on a quantitative analysis, which relied on $\pm$ 1.5 fold-change. Additionally, statistical significance was assessed using Student's *t*-test, Mann-Whitney Signed Rank test and Partial Least Squares test, with 95% confidence level. Protein spots obtained from quantitative and/or statistical analysis were considered differentially expressed between irradiated and non-irradiated macrophages.

Protein identification by mass spectrometry

The selected proteins were excised from Coomassie Blue-stained gels, with a spotpicker (OneTouch 2-DE gel spotpicker, Gel Company) and then processed for Matrix Assisted Laser Desorption/Ionization mass spectrometry (MALDI-TOF/TOF) analysis with trypsin in agreement with manufacturer´s instructions (Promega, USA). Therefore, protein digests were desalted, concentrated, and spotted onto a MALDI plate using reversed-phase (RP) C18 ZipTips (Millipore). The MALDI matrix was α-Cyano-4-hydroxycinnamic acid (8 mg/mL, 50% ACN, 0.1% TFA). Samples were analysed using a 4800 Plus MALDI TOF/TOF Analyser (SCIEX, Framingham, MA). Peptide mass fingerprint (PMF) data was collected in positive MS reflector mode, in the range of *m/z* 700−4000, and calibrated with external standards and trypsin autolysis peaks. The number of laser shots per MS1 scan was 2 000, while the number of laser shots per MS/MS was set between 2 000 and 4 000 in positive mode. The highest intensity MS peptide peaks were selected for MS/MS analysis in each spot. The number of precursors selected for tandem-MS varied between 7 and 17.

The MS and MS/MS spectra were processed and analysed using the software ProteinPilot (Version 4.5, SCIEX) and matched against the SwissProt (release 2014_05) protein sequence database using the Mascot search engine (Matrix Science) limited to *Homo sapiens* taxonomy, being the number of SwissProt sequences for the reference proteome of 40 550, following an already published procedure[30]. The search parameters were: Peptide Mass tolerance of 20 ppm; Fragment Mass tolerance of 1 Da; Trypsin maximum missed cleavages was set to 2; Fixed modification: Carbamidomethylation (Cysteine); Variable modifications: Oxidation (Methionine); and keratins were filtered out. Particularly, in the 2D gel spot "K", the protein type I cytoskeletal 9 keratin (P35527) was identified with a score of 65. The respective detected peptide ions were: *m/z* 745.41, *m/z* 1235.53, *m/z* 1323.67, *m/z* 1791.72, and *m/z* 1867.91. Since this protein is a typical external contaminant it was not considered as a valid identification. Protein scores greater than 59 were considered significant ($P < 0.05$) by the Mascot software (Matrix Science). No post processing baseline correction and smoothing was performed. Peptide peak lists were imported through Protein Pilot software (SCIEX). Protein identification was performed by the Mascot software and included the combined information of PMF and MS/MS peptide sequencing (Matrix Science). Tandem mass spectra were not processed. Proteins were identified using the combined information of PMF and MS/MS peptide sequencing. To be considered a match, a confidence interval, calculated by the Mascot software, of at least 95% was required.

Gel-free

Protein digestion and labelling with iTRAQ reagents

After protein extraction, about 1 mg of protein (*n* = 4) was precipitated with acetone (1:8) (v/v) and kept at -80°C for 15-20 min. Precipitated proteins were then centrifuged at 20 000 *g* for 15 min and resuspended in 0.5 M of Triethylammonium bicarbonate buffer (TEAB) (Sigma-Aldrich) pH 8.5, and vortexed. To better dissolve the pellet, samples were sonicated for 2 min in a cup horn at 20% and then 40% amplitude, 1 s ON and 1 s OFF cycle (Vibra Cell 750 watt, Sonics). Protein content was quantified using 2-D Quant Kit (GE Healthcare), according to manufacturer´s instructions. A volume correspondent to 100 μg of protein was concentrated in a rotary evaporator (Concentrator Plus, Eppendorf) at 60°C. Protein pellet was dissolved in TEAB 0.5 M to a final volume of 90 μL, and 8 μL of the reducing agent Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) 50 mM (Sigma-Aldrich) were added, vortexed to mix, spinned and sonicated in a cup horn for 1 min at 20% amplitude to facilitate protein denaturation. Then, 4 μL of 200 mM methyl methanethiosulfonate (MMTS) (Sigma-Aldrich) were added at room temperature, for 10 min, to block cysteins. TEAB 0.5 M was added to a final volume of 190 μL and the sample was vortexed. Protein digestion was performed by adding 10 μL of trypsin (Roche) (0.5 μg/μL), diluted in TEAB 0.5 M, to reach a 1:20 (w:w) enzyme:protein ratio, followed by incubation at 37°C overnight (16 h). After digestion, 2 μL of formic acid (FA) 100% were added to each sample and the sample was dried by rotary evaporation under vacuum for 1 h at 60°C. Samples were solubilized in 75 μL of 70% isopropanol/30% TEAB and sonicated for 10 min at 20% amplitude, with pulses of 1 s ON and 1 s OFF. Digested peptides were then labelled with the iTRAQ (8-plex) tags according to manufacturer´s instructions (Applied Biosystems, Foster City, CA) protocol. Briefly, peptides were added to each label and incubated for 2 h, at room temperature, and the reaction was stopped by incubation with 100 μL of water for 30 min. Eight samples from four blood donors were used in the 8-plex: 4 samples from irradiated (5 x 2 Gy) macrophages (labelled with 114, 116, 118, 121) and another 4 corresponding to non-irradiated macrophages (labelled with 113, 115, 117 and 119 reporter ions), which were then all combined into a single mixture.

Two-dimensional liquid chromatography (2D LC)-MS/MS

About 650 μg of peptide sample were solubilized in 2% acetonitrile (ACN) in 72mM TEAB and analysed by 2D LC-MS/MS, with a high pH reverse phase chromatography, as the first dimension, and then analysed by LC-MS. The first dimension chromatography was performed in Ultimate™3000 LC (LC Packings, Dionex) with two online Aeris 3.6 μm XB-C18 columns (15 cm x

2.10 mm) (Phenomenex), using 72 mM TEAB pH 8.5, as mobile phase A, and 72 mM TEAB in ACN pH 8.5, as mobile phase B (10 minutes with 2% mobile phase B followed by a linear gradient until 45% mobile phase B during 60 min, then followed by column wash and re-equilibration). Throughout the run, 74 fractions were collected, which were then joined into 19 samples, evaporated and prepared for LC-MS/MS. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent) on a ChromXP™ C18AR reverse phase column (300 μm ID x 15 cm length, 3 μm particles, 120 Å pore size, Eksigent) at 5 μL/min. Peptides were eluted into the mass spectrometer with an ACN gradient in 0.1% FA (2 to 30% ACN, in a linear gradient for 80 min, followed by a column wash and equilibration step), using an electrospray ionization source (DuoSpray™ Source, Sciex). The mass spectrometer (Triple TOF™ 5600 System, AB Sciex) was programmed for scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 30 MS/MS scans (100-1500 *m/z* for 100ms each). Candidate ions with a charge state between +2 and +5 and a minimum threshold of 70 counts/s were isolated for fragmentation and two MS/MS spectra were collected, before adding those ions to the exclusion list for 15 s (mass spectrometer operated by Analyst TF 1.6, Sciex). Specific iTRAQ rolling collision energy was used.

Database search and protein analysis

Peptide and protein identification and quantification was performed with ProteinPilot™ software (v4.5, ABSciex). Search parameters used were the following: SwissProt database (release 2012_06), against *Homo sapiens*, using MMTS alkylated cysteines and iTRAQ labelled peptides as fixed modifications. For data normalization, both Protein Pilot´s bias and background corrections were performed. The first allows for correction of systematic errors, due to unequal mixing of labelled samples by calculating the median protein ratio for all proteins reported in each sample, adjusted to unity, and assigning an autobias factor to it. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with ProteinPilot software was used to assess the quality of the identifications. Positive identifications were considered when identified proteins and peptides reached a confidence value > 95%[31] (5% local FDR), corresponding to a threshold cut-off of 2.01 (unused ProtScore). To increase the confidence level of quantified proteins, only those with at least 2 peptides used for quantification were considered for further comparative analysis. A maximum of one outlier value per quantified protein was excluded using Grub´s test (GraphPad Prism Software). Then, a statistical analysis, using one sample *t*-test, compared the protein value of irradiated macrophages (obtained through the four ratios: 114:113, 116:115, 118:117, 121:119) against a hypothetical value of "1". Additionally, a quantitative analysis, involving a 1.3-fold-change cut-off, as used by other authors[32,33], was performed. Proteins that

exhibit an iTRAQ ratio equal/larger than 1.3 (1 x 1.3) or equal/smaller than 0.77 (1 / 1.3), in at least 3 donors, were considered upregulated or downregulated, respectively. Only those proteins that passed both statistical and quantitative analysis were considered differentially expressed between irradiated and non-irradiated macrophages.

Bioinformatic analysis

Data from both gel-based and gel-free approaches was analysed with different bioinformatics tools. Enrichment analysis for the Gene Ontology categories was performed with WebGestalt software (WEB-based GEne SeT AnaLysis Toolkit) (version updated on 1/30/2013)[34,35], STRING (Search Tool for the Retrieval of Interacting Genes/*Proteins*) database (version 10.0)[36] and PANTHER (*Protein Analysis THrough Evolutionary* Relationships) Classification System (database version 6.1), using human genome as reference. The *heat map and cluster analysis were performed using GeneCluster (version 3.0) and Tree View (version 1.60) software*[37], while Venn diagrams were generated using the *Venny software*[38]*. Finally* protein-protein interaction networks were constructed using ClueGO+CluPedia plugins[23] of Cytoscape software (version 3.2.0)[22] and also STRING database.

Cell metabolic activity

To complement daily microscopic observation of irradiated and non-irradiated macrophages, macrophage metabolic activity was determined through the resazurin reduction assay. Briefly, 20 h after exposure to five cumulative ionizing radiation doses (5 x 2 Gy), macrophages were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich, MO, USA) for 3 h, at 37°C and 5% CO_2. After resazurin reduction, fluorescence was measured (530 nm Ex/590 nm Em), using the multi-mode microplate reader Synergy MX (BioTek, VT, USA).

Glucose uptake

Glucose levels were measured in conditioned medium (CM) from irradiated (5 x 2 Gy) and non-irradiated macrophages (n = 6), collected 24 h after irradiation. Briefly, CM was incubated with reagent 1 (mti-Diagnostics, Germany), composed of phosphate buffer, phenol, glucose oxidase, peroxidase and 4-Amino-antipyrine, for 20 min at room temperature. In the first step, glucose was converted into D-glucono-1,5-lactone plus hydrogen peroxide by glucose oxidase, which was then used for the second step, in which peroxidase generated a coloured product. The colour intensity was proportional to the glucose concentration in the sample. The absorbance was read at 500 nm with the multi-mode microplate reader Synergy MX (BioTek). The glucose concentration values were subtracted to that of RPMI 1640 medium, which is equivalent to 11.11 nM, to obtain glucose

uptake levels. Data from irradiated macrophages was compared to that of non-irradiated ones and expressed as fold-change.

Lactate levels

Briefly, 24 h after irradiation, CM from irradiated (5 x 2 Gy) macrophages and their counterparts (*n* = 6) was collected and incubated with working reagent (Spinreact, Spain), composed of PIPES buffer, 4-Chlorophenol, lactate oxidase, peroxidase and 4-Aminophenazone, for 10 min at room temperature. Lactate was then oxidized by lactate oxidase to pyruvate and hydrogen peroxide, which under the presence of peroxidase, and the remaining reagent compounds, formed a red quinone product. The intensity of the colour formed was proportional to the lactate concentration in the sample. The absorbance was read at 505 nm with the multi-mode microplate reader Synergy MX (BioTek). A lactate standard (1.123 nmol/L) was used as a reference value. Finally, data was normalized to CM protein concentration and lactate levels of irradiated macrophages were then compared to that of non-irradiated ones and expressed as fold-change.

Total levels of cellular ATP

Total levels of cellular ATP were measured with Luminescent ATP Detection Assay Kit (MitoSciences, OR), according to manufacturer´s instructions. Briefly, 24 h after irradiation (5 x 2 Gy), irradiated macrophages and their counterparts (*n* = 6) were lysed with detergent, allowing ATPases to be irreversibly inactivated, and incubated with substrate solution, containing luciferase and D-luciferin, which react with ATP. The emitted light was proportional to the ATP concentration inside the cell. Luminescence was then measured with the multi-mode microplate reader Synergy MX (BioTek). ATP values of each sample were obtained from a standard curve previously performed with ATP dilution series and normalized to CM protein concentration.

Western Blot

For evaluation of radiation-induced DNA damage, macrophage protein lysates were evaluated for H2AX phosphorylation, through western blot analysis. Briefly, proteins were extracted with Laemmli buffer 1x (3% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.1% blue bromophenol in 1M Tris-HCl pH 6.8), about 40 min after irradiation (5 x 2 Gy). Macrophage lysates (*n* = 3) were sonicated for 5 s to shear DNA and, heated at 95°C for 5 min, and 5 µg were loaded on 15% SDS-polyacrylamide gels. Proteins were separated by electrophoresis, and transferred onto nitrocellulose membrane (GE Healthcare, UK). Membranes were blocked in 5% powered milk, diluted in PBS-Tween 0.5%, for 1 h. Incubation with primary antibody against histone-H2AX (Ser139) (γH2AX) (clone JBW301) (Millipore, Germany) was performed overnight, at 4°C. Antibody

against α-tubulin (Sigma-Aldrich) was used to normalize protein expression. Sheep anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) was used for 1 h, at room temperature, followed by ECL detection (GE Healthcare, UK). Protein bands were quantified using Quantity One software (version 4.6.5, BioRad).

In order to validate the expression of cathepsin D, V-type proton ATPase subunit D and transferrin receptor, macrophage proteins were extracted with RIPA buffer, composed as described in *Protein extraction* subsection. About 25 µg of protein were diluted in Laemmli buffer containing β-mercaptoethanol (BioRad), denatured at 95°C and loaded in 10-15% SDS-polyacrylamide gels. Western blot was performed as above described, excepting that primary antibodies against cathepsin D (clone BC011) (Millipore), V-type proton ATPase subunit D (GeneTex) or transferrin receptor protein 1 (Novocastra), were used. For V-type proton ATPase subunit D, an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) was used.

Mathematical network

The methodology used was based on the quantitative data, i.e. the expression ratios for the 67 proteins considered altered between irradiated (5 x 2 Gy) and non-irradiated macrophages (n = 4), obtained through gel-free analysis. This data was transformed in order to be able to constitute an edge list, as previously documented[39]. The resulting edge list was then used as input in the Gephi software to produce the measurements and models defined in the profiling framework[39]. This is an exclusively mathematical approach, which does not contemplate the biological meaning of the predicted associations found between nodes (proteins). Proteins were identified by their accession code, and protein increase upon macrophage irradiation (fold-change >= 1.3) was indicated by “accession code_I”, while protein decrease (fold-change <= 0.77) was indicated by “accession code_D”.

Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v5 (GraphPad-trial version). One sample or paired *t*-tests were used to test the hypothesis that irradiated macrophages were different from non-irradiated ones. Statistical significance was achieved when $P < 0.05$.

References

1. Delaney, G.; Jacob, S.; Featherstone, C.; Barton, M., The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer 2005, 104 (6), 1129-37.
2. Ringborg, U.; Bergqvist, D.; Brorsson, B.; Cavallin-Stahl, E.; Ceberg, J.; Einhorn, N.; Frodin, J. E.; Jarhult, J.; Lamnevik, G.; Lindholm, C.; Littbrand, B.; Norlund, A.; Nylen, U.; Rosen, M.; Svensson, H.; Moller, T. R., The Swedish Council on Technology Assessment in Health Care (SBU) systematic overview of radiotherapy for cancer including a prospective survey of radiotherapy practice in Sweden 2001--summary and conclusions. Acta Oncol 2003, 42 (5-6), 357-65.
3. Allen, B. J.; Bezak, E.; Marcu, L. G., Quo vadis radiotherapy? Technological advances and the rising problems in cancer management. Biomed Res Int 2013, 2013, 749203.
4. Bernier, J.; Hall, E. J.; Giaccia, A., Radiation oncology: a century of achievements. Nat Rev Cancer 2004, 4 (9), 737-47.
5. Hofmeister, V.; Schrama, D.; Becker, J. C., Anti-cancer therapies targeting the tumor stroma. Cancer Immunol Immunother 2008, 57 (1), 1-17.
6. Jain, R. K., Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J Clin Oncol 2013, 31 (17), 2205-18.
7. Madani, I.; De Neve, W.; Mareel, M., Does ionizing radiation stimulate cancer invasion and metastasis? Bull Cancer 2008, 95 (3), 292-300.
8. McBride, W. H.; Chiang, C. S.; Olson, J. L.; Wang, C. C.; Hong, J. H.; Pajonk, F.; Dougherty, G. J.; Iwamoto, K. S.; Pervan, M.; Liao, Y. P., A sense of danger from radiation. Radiat Res 2004, 162 (1), 1-19.
9. Davies, L. C.; Taylor, P. R., Tissue-resident macrophages: then and now. Immunology 2015, 144 (4), 541-8.
10. Murray, P. J.; Wynn, T. A., Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011, 11 (11), 723-37.
11. Lauber, K.; Ernst, A.; Orth, M.; Herrmann, M.; Belka, C., Dying cell clearance and its impact on the outcome of tumor radiotherapy. Front Oncol 2012, 2, 116.
12. Joyce, J. A.; Pollard, J. W., Microenvironmental regulation of metastasis. Nat Rev Cancer 2009, 9 (4), 239-52.
13. Condeelis, J.; Pollard, J. W., Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006, 124 (2), 263-6.
14. De Palma, M.; Lewis, C. E., Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell 2013, 23 (3), 277-86.
15. Mantovani, A.; Sica, A., Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 2010, 22 (2), 231-7.
16. Barker, H. E.; Paget, J. T.; Khan, A. A.; Harrington, K. J., The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer 2015, 15 (7), 409-25.
17. Heylmann, D.; Rodel, F.; Kindler, T.; Kaina, B., Radiation sensitivity of human and murine peripheral blood lymphocytes, stem and progenitor cells. Biochim Biophys Acta 2014, 1846 (1), 121-9.
18. Pinto, A. T.; Pinto, M. L.; Cardoso, A. P.; Monteiro, C.; Pinto, M. T.; Maia, A. F.; Castro, P.; Figueira, R.; Monteiro, A.; Marques, M.; Mareel, M.; Dos Santos, S. G.; Seruca, R.; Barbosa, M. A.; Rocha, S.; Oliveira, M. J., Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. Sci Rep 2016, 6, 18765.
19. College, O., Medical applications of nuclear physics. In College Physics, College, O., Ed. 2012; Vol. 3.
20. Frischholz, B.; Wunderlich, R.; Ruhle, P. F.; Schorn, C.; Rodel, F.; Keilholz, L.; Fietkau, R.; Gaipl, U. S.; Frey, B., Reduced secretion of the inflammatory cytokine IL-1beta by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation. Autoimmunity 2013, 46 (5), 323-8.
21. Tsukimoto, M.; Homma, T.; Mutou, Y.; Kojima, S., 0.5 Gy gamma radiation suppresses production of TNF-alpha through up-regulation of MKP-1 in mouse macrophage RAW264.7 cells. Radiat Res 2009, 171 (2), 219-24.
22. UNSCEAR, Biological mechanisms of radiation actions at low doses. A white paper to guide the Scientific Committee's future programme of work . United Nations: New York, 2012.
23. Wunderlich, R.; Ernst, A.; Rodel, F.; Fietkau, R.; Ott, O.; Lauber, K.; Frey, B.; Gaipl, U. S., Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. Clin Exp Immunol 2015, 179 (1), 50-61.
24. Hellevik, T.; Martinez-Zubiaurre, I., Radiotherapy and the tumor stroma: the importance of dose and fractionation. Front Oncol 2014, 4, 1.
25. Azimzadeh, O.; Atkinson, M. J.; Tapio, S., Proteomics in radiation research: present status and future perspectives. Radiat Environ Biophys 2014, 53 (1), 31-8.
26. Stulik, J.; Koupilova, K.; Hernychova, L.; Macela, A.; Blaha, V.; Baaske, C.; Kaffenberger, W.; van Beuningen, D., Modulation of signal transduction pathways and global protein composition of macrophages by ionizing radiation. Electrophoresis 1999, 20 (4-5), 962-8.
27. Chen, C.; Boylan, M. T.; Evans, C. A.; Whetton, A. D.; Wright, E. G., Application of two-dimensional difference gel electrophoresis to studying bone marrow macrophages and their *in vivo* responses to ionizing radiation. J Proteome Res 2005, 4 (4), 1371-80.

28. Smallwood, H. S.; Lopez-Ferrer, D.; Eberlein, P. E.; Watson, D. J.; Squier, T. C., Calmodulin mediates DNA repair pathways involving H2AX in response to low-dose radiation exposure of RAW 264.7 macrophages. Chem Res Toxicol 2009, 22 (3), 460-70.
29. Cardoso, A. P.; Pinto, M. L.; Pinto, A. T.; Oliveira, M. I.; Pinto, M. T.; Goncalves, R.; Relvas, J. B.; Figueiredo, C.; Seruca, R.; Mantovani, A.; Mareel, M.; Barbosa, M. A.; Oliveira, M. J., Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. Oncogene 2014, 33 (16), 2123-33.
30. Gomes, C.; Almeida, A.; Ferreira, J. A.; Silva, L.; Santos-Sousa, H.; Pinto-de-Sousa, J.; Santos, L. L.; Amado, F.; Schwientek, T.; Levery, S. B.; Mandel, U.; Clausen, H.; David, L.; Reis, C. A.; Osorio, H., Glycoproteomic analysis of serum from patients with gastric precancerous lesions. J Proteome Res 2013, 12 (3), 1454-66.
31. Song, X.; Bandow, J.; Sherman, J.; Baker, J. D.; Brown, P. W.; McDowell, M. T.; Molloy, M. P., iTRAQ experimental design for plasma biomarker discovery. J Proteome Res 2008, 7 (7), 2952-8.
32. Rukmangadachar, L. A.; Makharia, G. K.; Mishra, A.; Das, P.; Hariprasad, G.; Srinivasan, A.; Gupta, S. D.; Ahuja, V.; Acharya, S. K., Proteome analysis of the macroscopically affected colonic mucosa of Crohn's disease and intestinal tuberculosis. Sci Rep 2016, 6, 23162.
33. Ruppen, I.; Grau, L.; Orenes-Pinero, E.; Ashman, K.; Gil, M.; Algaba, F.; Bellmunt, J.; Sanchez-Carbayo, M., Differential protein expression profiling by iTRAQ-two-dimensional LC-MS/MS of human bladder cancer EJ138 cells transfected with the metastasis suppressor KiSS-1 gene. Mol Cell Proteomics 2010, 9 (10), 2276-91.
34. Zhang, B.; Kirov, S.; Snoddy, J., WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Res 2005, 33 (Web Server issue), W741-8.
35. Wang, J.; Duncan, D.; Shi, Z.; Zhang, B., WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. Nucleic Acids Res 2013, 41 (Web Server issue), W77-83.
36. Franceschini, A.; Szklarczyk, D.; Frankild, S.; Kuhn, M.; Simonovic, M.; Roth, A.; Lin, J.; Minguez, P.; Bork, P.; von Mering, C.; Jensen, L. J., STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res 2013, 41 (Database issue), D808-15.
37. Eisen, M. B.; Spellman, P. T.; Brown, P. O.; Botstein, D., Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998, 95 (25), 14863-8.
38. Oliveros, J. C. VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.
39. Sousa, J. L. R.; Machado, R. J., Sociomaterial Enactment Drive of Business/IT Alignment: From Small Data to Big Impact. Procedia Technology 2014, 16, 569-582.
40. Binns, D.; Dimmer, E.; Huntley, R.; Barrell, D.; O'Donovan, C.; Apweiler, R., QuickGO: a web-based tool for Gene Ontology searching. Bioinformatics 2009, 25 (22), 3045-6.
41. Mah, L. J.; El-Osta, A.; Karagiannis, T. C., gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia 2010, 24 (4), 679-86.
42. Anoopkumar-Dukie, S.; Carey, J. B.; Conere, T.; O'Sullivan, E.; van Pelt, F. N.; Allshire, A., Resazurin assay of radiation response in cultured cells. Br J Radiol 2005, 78 (934), 945-7.
43. Augustin, W.; Trumper, L.; Spengler, V., Metabolism of intact reticulocytes and mitochondria under lowered energy load induced by cycloheximide. Biomed Biochim Acta 1987, 46 (2-3), S229-33.
44. Bernstein, B. W.; Bamburg, J. R., Actin-ATP hydrolysis is a major energy drain for neurons. J Neurosci 2003, 23 (1), 1-6.
45. Bonora, M.; Patergnani, S.; Rimessi, A.; De Marchi, E.; Suski, J. M.; Bononi, A.; Giorgi, C.; Marchi, S.; Missiroli, S.; Poletti, F.; Wieckowski, M. R.; Pinton, P., ATP synthesis and storage. Purinergic Signal 2012, 8 (3), 343-57.
46. Lodish, H.; Berk, A.; SL, Z.; Matsudaira, P.; Baltimore, D.; Darnell, J., Section 15.5Active Transport by ATP-Powered Pumps. In Molecular Cell Biology, 4th edition ed.; W. H. Freeman: New York, 2000.
47. Gieselmann, V.; Pohlmann, R.; Hasilik, A.; Von Figura, K., Biosynthesis and transport of cathepsin D in cultured human fibroblasts. J Cell Biol 1983, 97 (1), 1-5.
48. Minarowska, A.; Gacko, M.; Karwowska, A.; Minarowski, L., Human cathepsin D. Folia Histochem Cytobiol 2008, 46 (1), 23-38.
49. Minarowska, A.; Karwowska, A.; Gacko, M., Quantitative determination and localization of cathepsin D and its inhibitors. Folia Histochem Cytobiol 2009, 47 (2), 153-77.
50. Rijnboutt, S.; Stoorvogel, W.; Geuze, H. J.; Strous, G. J., Identification of subcellular compartments involved in biosynthetic processing of cathepsin D. J Biol Chem 1992, 267 (22), 15665-72.
51. Cervelli, T.; Panetta, D.; Navarra, T.; Andreassi, M. G.; Basta, G.; Galli, A.; Salvadori, P. A.; Picano, E.; Del Turco, S., Effects of single and fractionated low-dose irradiation on vascular endothelial cells. Atherosclerosis 2014, 235 (2), 510-8.
52. Mestas, J.; Hughes, C. C., Of mice and not men: differences between mouse and human immunology. J Immunol 2004, 172 (5), 2731-8.
53. Kato, T.; Kojima, K.; Murachi, T., Proteases of macrophages in rat peritoneal exudate, with special reference to the effects of actinomycete protease inhibitors. Biochim Biophys Acta 1972, 289 (1), 187-93.
54. Aderem, A.; Underhill, D. M., Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 1999, 17, 593-623.
55. Benes, P.; Vetvicka, V.; Fusek, M., Cathepsin D--many functions of one aspartic protease. Crit Rev Oncol Hematol 2008, 68 (1), 12-28.

56. Trost, M.; English, L.; Lemieux, S.; Courcelles, M.; Desjardins, M.; Thibault, P., The phagosomal proteome in interferon-gamma-activated macrophages. Immunity 2009, 30 (1), 143-54.
57. Yates, R. M.; Hermetter, A.; Taylor, G. A.; Russell, D. G., Macrophage activation downregulates the degradative capacity of the phagosome. Traffic 2007, 8 (3), 241-50.
58. Adams, D. O.; Hamilton, T. A., The cell biology of macrophage activation. Annu Rev Immunol 1984, 2, 283-318.
59. Balce, D. R.; Li, B.; Allan, E. R.; Rybicka, J. M.; Krohn, R. M.; Yates, R. M., Alternative activation of macrophages by IL-4 enhances the proteolytic capacity of their phagosomes through synergistic mechanisms. Blood 2011, 118 (15), 4199-208.
60. Canton, J.; Khezri, R.; Glogauer, M.; Grinstein, S., Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages. Mol Biol Cell 2014, 25 (21), 3330-41.
61. Delamarre, L.; Couture, R.; Mellman, I.; Trombetta, E. S., Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. J Exp Med 2006, 203 (9), 2049-55.
62. Boya, P.; Kroemer, G., Lysosomal membrane permeabilization in cell death. Oncogene 2008, 27 (50), 6434-51.
63. Appelqvist, H.; Johansson, A. C.; Linderoth, E.; Johansson, U.; Antonsson, B.; Steinfeld, R.; Kagedal, K.; Ollinger, K., Lysosome-mediated apoptosis is associated with cathepsin D-specific processing of bid at Phe24, Trp48, and Phe183. Ann Clin Lab Sci 2012, 42 (3), 231-42.
64. Gogvadze, V.; Orrenius, S.; Zhivotovsky, B., Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta 2006, 1757 (5-6), 639-47.
65. Roberg, K.; Kagedal, K.; Ollinger, K., Microinjection of cathepsin d induces caspase-dependent apoptosis in fibroblasts. Am J Pathol 2002, 161 (1), 89-96.
66. Bewley, M. A.; Pham, T. K.; Marriott, H. M.; Noirel, J.; Chu, H. P.; Ow, S. Y.; Ryazanov, A. G.; Read, R. C.; Whyte, M. K.; Chain, B.; Wright, P. C.; Dockrell, D. H., Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to Streptococcus pneumoniae. Mol Cell Proteomics 2011, 10 (6), M111 008193.
67. Davydov, D. R., Microsomal monooxygenase in apoptosis: another target for cytochrome c signaling? Trends Biochem Sci 2001, 26 (3), 155-60.
68. Mauk, A. G.; Mauk, M. R.; Moore, G. R.; Northrup, S. H., Experimental and theoretical analysis of the interaction between cytochrome c and cytochrome b5. J Bioenerg Biomembr 1995, 27 (3), 311-30.
69. Bryan, N. B.; Dorfleutner, A.; Rojanasakul, Y.; Stehlik, C., Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain. J Immunol 2009, 182 (5), 3173-82.
70. Ohtsuka, T.; Ryu, H.; Minamishima, Y. A.; Macip, S.; Sagara, J.; Nakayama, K. I.; Aaronson, S. A.; Lee, S. W., ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. Nat Cell Biol 2004, 6 (2), 121-8.
71. Kim, M. H.; Jung, S. Y.; Ahn, J.; Hwang, S. G.; Woo, H. J.; An, S.; Nam, S. Y.; Lim, D. S.; Song, J. Y., Quantitative proteomic analysis of single or fractionated radiation-induced proteins in human breast cancer MDA-MB-231 cells. Cell & bioscience 2015, 5, 2.
72. Vatner, R. E.; Formenti, S. C., Myeloid-derived cells in tumors: effects of radiation. Semin Radiat Oncol 2015, 25 (1), 18-27.
73. Wang, X.; Belguise, K.; O'Neill, C. F.; Sanchez-Morgan, N.; Romagnoli, M.; Eddy, S. F.; Mineva, N. D.; Yu, Z.; Min, C.; Trinkaus-Randall, V.; Chalbos, D.; Sonenshein, G. E., RelB NF-kappaB represses estrogen receptor alpha expression via induction of the zinc finger protein Blimp1. Mol Cell Biol 2009, 29 (14), 3832-44.
74. Kong, W. N.; Lei, Y. H.; Chang, Y. Z., The regulation of iron metabolism in the mononuclear phagocyte system. Expert review of hematology 2013, 6 (4), 411-8.
75. Pan, X.; Tamilselvam, B.; Hansen, E. J.; Daefler, S., Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens. BMC microbiology 2010, 10, 64.
76. Corna, G.; Campana, L.; Pignatti, E.; Castiglioni, A.; Tagliafico, E.; Bosurgi, L.; Campanella, A.; Brunelli, S.; Manfredi, A. A.; Apostoli, P.; Silvestri, L.; Camaschella, C.; Rovere-Querini, P., Polarization dictates iron handling by inflammatory and alternatively activated macrophages. Haematologica 2010, 95 (11), 1814-22.
77. Kenneth, N. S.; Mudie, S.; Naron, S.; Rocha, S., TfR1 interacts with the IKK complex and is involved in IKK-NF-kappaB signalling. Biochem J 2013, 449 (1), 275-84.
78. Harrington, N. P.; Chambers, K. A.; Ross, W. M.; Filion, L. G., Radiation damage and immune suppression in splenic mononuclear cell populations. Clin Exp Immunol 1997, 107 (2), 417-24.
79. Chen, Z.; Nomura, J.; Suzuki, T.; Suzuki, N., Enhanced expression of transferrin receptor confers UV-resistance in human and monkey cells. Journal of radiation research 2005, 46 (4), 443-51.
80. Vieira, O. V.; Botelho, R. J.; Grinstein, S., Phagosome maturation: aging gracefully. Biochem J 2002, 366 (Pt 3), 689-704.
81. Fuller, H. R.; Morris, G. E., Quantitative Proteomics Using iTRAQ Labeling and Mass Spectrometry. In Integrative Proteomics, Leung, D. H.-C., Ed. InTech: 2012.

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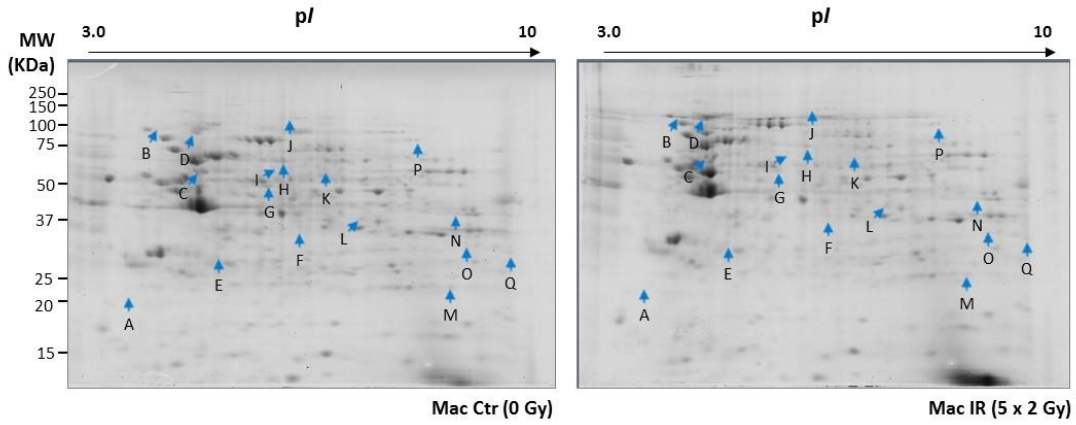
Supporting Information

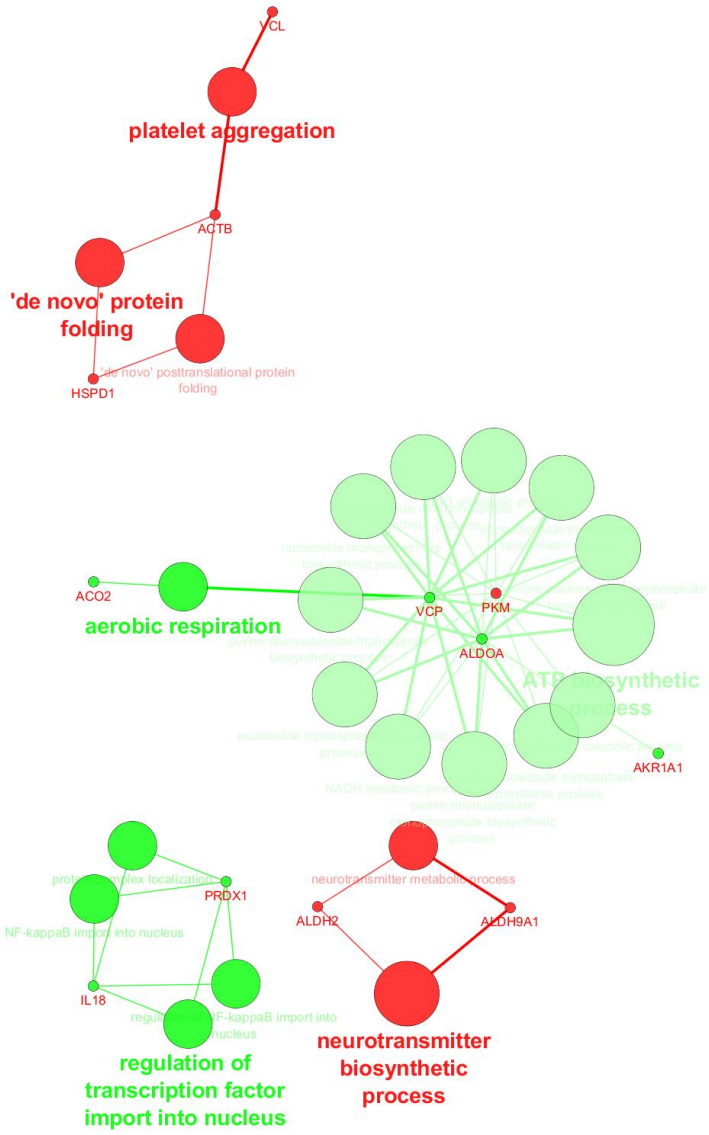
SUPPLEMENTARY FIGURES:



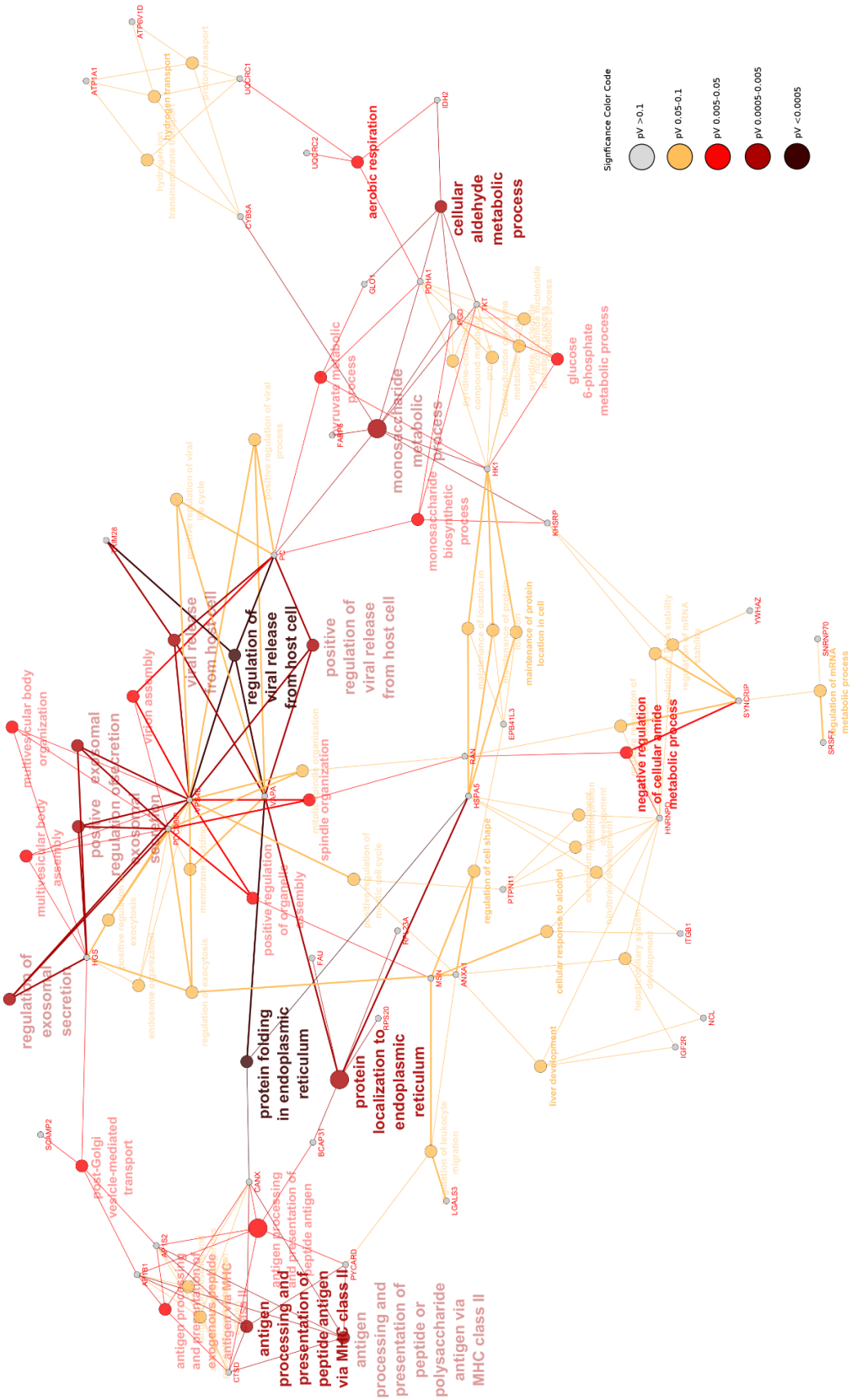
Supplementary Figure S1 - Detection of proteins spots differentially altered between irradiated (Mac 5 x 2 Gy) and non-irradiated macrophages (Mac 0 Gy) (*n* = 7), obtained through 2-DE approach. Representative images of gels from non-irradiated (*left*) and irradiated macrophages (*right*) are presented. Protein molecular weights (MW) are indicated on the left side, while protein isoelectric points (pI) are annotated on the top of each gel. Differentially expressed proteins are marked with an arrow and identified with a letter (A-Q).

Note: Due to space limitation, it is not possible to present Supplementary Figure S2.

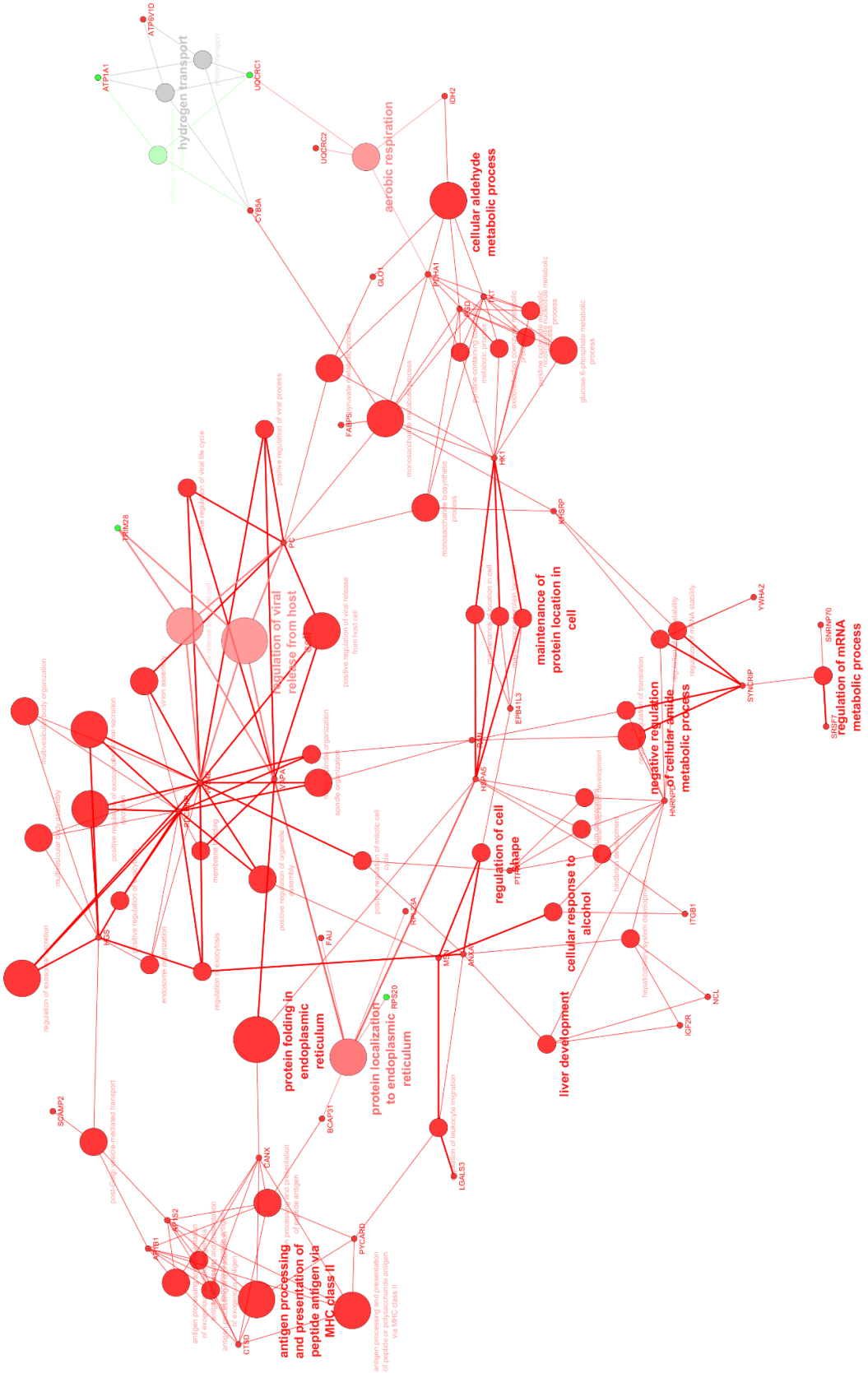
Supplementary Figure S2 – Gene ontology (GO) enrichment analysis regarding biological processes, molecular functions and cellular components of 17 proteins differentially expressed, corresponding to 23 protein IDs, obtained with gel-based approach (Web-based Gene Set Analysis Toolkit). GO categories in the top 10 are presented, being those with a significant enrichment ($P < 0.05$) written in red.



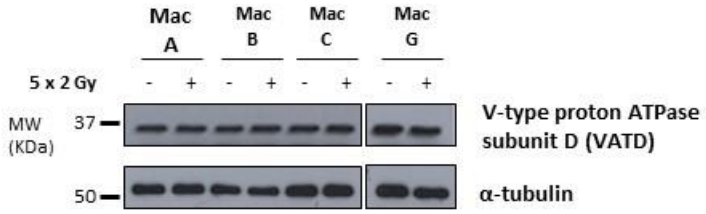
Supplementary Figure S3. ClueGo/CluPedia analysis of protein-protein interaction considering proteins differentially expressed between irradiated (5 x 2 Gy) and non-irradiated macrophages, through gel-based approach (Cytoscape version 3.2.0). Green nodes refer to biological processes positively regulated, whereas red nodes refer to the ones negatively regulated in irradiated macrophages.



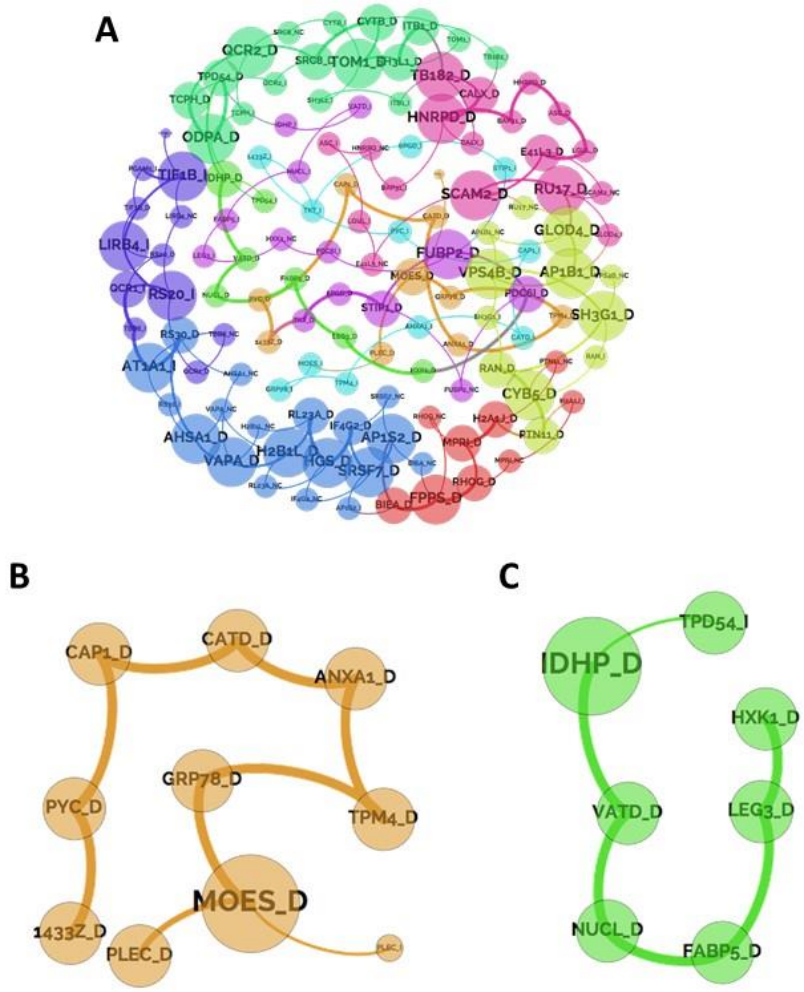
Supplementary Figure S4 – ClueGo/CluPedia analysis of protein-protein interactions considering the biological processes significantly associated with the altered targets, which were identified by gel-free approach, in irradiated (5 x 2 Gy) macrophages (Cytoscape version 3.2.0). Dark nodes refer to the most significant associations.



Supplementary Figure S5 – ClueGo/CluPedia analysis of protein-protein interaction considering proteins differentially expressed between irradiated (5 x 2 Gy) and non-irradiated macrophages, through gel-free approach (Cytoscape version 3.2.0). Green nodes refer to biological processes positively regulated, whereas red nodes refer to the ones negatively regulated in irradiated macrophages.



Supplementary Figure S6 - V-type proton ATPase subunit D does not seem to be downregulated in irradiated (5 x 2 Gy) macrophages. VATD expression was evaluated by western blot analysis in irradiated and non-irradiated macrophages from 4 blood donors used in gel-based or gel-free proteomic approaches.



Supplementary Figure S7 – Protein-protein interaction networks involving cathepsin D and V-type proton ATPase subunit D. A mathematical model was used to correlate fold-change alterations between proteins. **A)** The degree topology of proteins, identified through gel-free approach (see targets in Supplementary Table S6), which expression was altered in macrophages upon ionizing radiation exposure revealed, by different colours, the emergence of 10 communities. The degree topology of the communities where the downregulation of **B)** cathepsin D (CATD_D) or **C)** V-type proton ATPase subunit D (VATD_D) belongs are also presented. When visually evaluating the degree, its node size positively correlates with its degree and a strongest pathway between nodes indicates a stronger relation between proteins. Protein names: ANXA1

- annexin A1, CAP1 - adenylyl cyclase-associated protein 1, CATD - cathepsin D, FABP5 - fatty acid-binding protein, GRP78 - 78 kDa glucose-regulated protein, HXK1 - hexokinase-1, IDHP - isocitrate dehydrogenase [NADP], LEG - galectin-3, MOES – moesin, NUCL – nucleolin, PLEC - plectin, PYC - pyruvate carboxylase, TDP54 - tumor protein D54, TPM4 - tropomyosin alpha-4 chain, VATD - V-type proton ATPase subunit D, 1433Z - 14-3-3 protein zeta/delta.

SUPPLEMENTARY TABLES:

Note: Due to space limitation, only the most relevant data of Supplementary Table S1 and Supplementary Table S6 are possible to present here.

Supplementary Table S1 - List of differentially expressed proteins in irradiated (5 x 2 Gy) human macrophages, identified through gel-based approach.

Spot code	Gene name	Protein name	UniProtKB-ID		Protein Score	Sequence lengh (aminoacids)	Protein sequence coverage (%)	observed / expected MW	observed / expected pI	Searched /Matched PMF spectra	Matched MS/MS spectra	Unique peptides assigned	Expected score	Ratio (7 IR / 7 Ctr)
			Accession number	Entry name										
A	IL18	Interleukin-18	Q14116	IL18	130	193	26	21/22.6	3.3/4.54	77/8	4	4	4,10E-09	1.36
B	USP5	Ubiquitin carboxyl-terminal hydrolase 5	P45974	UBP5	108	858	9	94/96.6	4.1/4.91	80/10	3	3	5,10E-07	0.47
C	HSPD1	60 kDa heat shock protein, mitochondrial	P10809	CH60	482	573	27	58/61.2	5/5.70	77/16	7	5	2,60E-44	0.71
	ACTB	Actin, cytoplasmic 1	P60709	ACTB	295	375	20	58/42.0	5/5.29	77/10	4	3	1,30E-25	
D	VCP	Transitional endoplasmic reticulum ATPase	P55072	TERA	466	806	22	88/89.9	5/5.14	81/27	8	6	1,00E-42	1.39
E	CTSD	Cathepsin D	P07339	CATD	319	412	23	29/26.6[a)]	5.1/5.56[a)]	82/12	4	4	5,10E-28	1.68
F	ANXA2	Annexin A2	P07355	ANXA2	304	339	58	35/38.8	6/7.57	75/24	5	5	1,60E-26	0.62
G	CNDP2	Cytosolic non-specific dipeptidase	Q96KP4	CNDP2	313	475	20	50/53.2	5.8/5.66	77/14	3	3	2,00E-27	0.79
	ALDH2	Aldehyde dehydrogenase, mitochondrial	P05091	ALDH2	196	517	9	50/56.9	5.8/6.63	77/7	4	4	1,00E-15	
	ALDH9A1	4-trimethylaminobutyraldehyde dehydrogenase	P49189	AL9A1	66	494	12	50/54.7	5.8/5.69	77/8	2	2	0,0091	
H	LTA4H	Leukotriene A-4 hydrolase	P09960	LKHA4	462	611	40	65/69.9	5.9/5.80	72/32	6	5	2,60E-42	0.65
I	ME1	NADP-dependent malic enzyme	P48163	MAOX	129	572	12	60/64.7	5.9/5.79	73/13	4	4	5,10E-09	0.64
J	VCL	Vinculin	P18206	VINC	157	1134	24	118/124.2	5.9/5.50	69/31	2	2	8,10E-12	0.54
K	PKM	Pyruvate kinase PKM	P14618	KPYM	144	531	19	59/58.5	6.1/7.96	74/12	2	2	1,60E-10	0.52
L	AKR1A1	Alcohol dehydrogenase [NADP(+)]	P14550	AK1A1	317	325	40	37/36.9	6.2/6.32	77/20	4	4	8-1e-28	1.43
	ACAT2	Acetyl-CoA acetyltransferase, cytosolic	Q9BWD1	THIC	176	397	14	37/41.8	6.2/6.47	77/9	3	3	1,00E-13	
M	PRDX1	Peroxiredoxin-1	Q06830	PRDX1	376	199	64	23/22.3	8.1/8.27	76/20	3	3	1,00E-33	1.93
	CD48	CD48 antigen	P09326	CD48	61	243	9	23/28.1	8.1/8.34	76/2	1	1	0,031	
N	ALDOA	Fructose-bisphosphate aldolase A	P04075	ALDOA	115	364	15	38/39.8	8.3/8.30	72/5	2	2	1,30E-07	1.86
O	VDAC1	Voltage-dependent anion-selective channel protein 1	P21796	VDAC1	98	283	11	31/30.9	8.6/8.62	72/3	2	2	6,90E-06	0.7
	ANXA2	Annexin A2	P07355	ANXA2	93	339	10	31/38.8	8.6/7.57	72/3	2	2	2,00E-05	
P	ACO2	Aconitate hydratase, mitochondrial	Q99798	ACON	283	780	14	79/86.1	7.2/7.36	74/12	3	3	2,00E-24	1.64
Q	ATP6V1D	V-type proton ATPase subunit D	Q9Y5K8	VATD	118	247	14	30/28.2	9.5/9.36	74/4	3	3	6,40E-08	0.79

[a)] Although the molecular weigh of cathepsin D is 45 KDa, it can be cleaved into two chains that consequently exhibit a lower molecular weight. Accordingly, MALDI data indicates the identification of cathepsin D heavy chain, which presents the molecular weight and isoelectric point mentioned in the table.

Supplementary Table S2 - List of proteins identified in irradiated (5 x 2 Gy) and non-irradiated macrophages through gel-free approach.

Supplementary Table S3 - Detailed information on the peptides used to identify proteins obtained from gel-free approach.

Supplementary Table S4 - List of proteins quantified in irradiated (5 x 2 Gy) and non-irradiated macrophages, obtained through gel-free approach.

Supplementary Table S5 - Detailed information on the peptides used to identify proteins obtained from gel-free approach.

Supplementary Table S6 – List of up and downregulated targets in macrophages exposed to cumulative ionizing radiation doses (5 x 2 Gy), compared with non-irradiated ones, and obtained through gel-free approach.

						Donor D	Donor E	Donor F	Donor G
Accession Name	Accession Name	Molecular Weight	Unique Peptides for ID (95% FDR)	Peptides used for Quant	Unique Peptides used for Quant	114:113	116:115	118:117	121:119
PLEC	Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3	531,79	118,00	217,00	94,00	0,51	0,69	0,66	3,60
MOES	Moesin OS=Homo sapiens GN=MSN PE=1 SV=3	67,82	41,00	190,00	32,00	0,37	9,12	0,35	0,58
GRP78	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2	72,33	38,00	196,00	29,00	0,59	4,21	0,53	0,44
TPM4	Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3	28,52	36,00	168,00	15,00	0,30	7,87	0,34	0,40
ANXA1	Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	38,71	30,00	281,00	25,00	0,63	2,33	0,33	0,66
CATD	Cathepsin D OS=Homo sapiens GN=CTSD PE=1 SV=1	44,55	24,00	55,00	15,00	0,52	6,03	0,55	0,13
CAP1	Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=5	51,90	22,00	108,00	17,00	0,69	1,64	0,48	0,61
PYC	Pyruvate carboxylase, mitochondrial OS=Homo sapiens GN=PC PE=1 SV=2	129,63	21,00	25,00	12,00	0,75	1,63	0,44	0,63
1433Z	14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1	27,75	20,00	54,00	13,00	0,51	2,07	0,65	0,46
TKT	Transketolase OS=Homo sapiens GN=TKT PE=1 SV=3	67,88	20,00	47,00	18,00	0,23	2,54	0,37	0,39
6PGD	6-phosphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE=1 SV=3	53,14	17,00	120,00	12,00	0,56	2,63	0,73	0,38
STIP1	Stress-induced-phosphoprotein 1 OS=Homo sapiens GN=STIP1 PE=1 SV=1	62,64	16,00	90,00	17,00	0,44	1,41	0,72	0,60
FUBP2	Far upstream element-binding protein 2 OS=Homo sapiens GN=KHSRP PE=1 SV=4	73,12	16,00	12,00	7,00	0,60	0,72	0,86	0,69
PDC6I	Programmed cell death 6-interacting protein OS=Homo sapiens GN=PDCD6IP PE=1 SV=1	96,02	15,00	36,00	13,00	0,49	1,45	0,62	0,71
HXK1	Hexokinase-1 OS=Homo sapiens GN=HK1 PE=1 SV=3	102,49	14,00	22,00	9,00	0,59	0,81	0,47	0,58
LEG3	Galectin-3 OS=Homo sapiens GN=LGALS3 PE=1 SV=5	26,15	13,00	44,00	7,00	0,66	8,24	0,52	0,36
FABP5	Fatty acid-binding protein, epidermal OS=Homo sapiens GN=FABP5 PE=1 SV=3	15,16	12,00	72,00	9,00	0,74	7,24	0,61	0,52
NUCL	Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3	76,61	10,00	25,00	14,00	0,40	2,44	0,67	0,61
VATD	V-type proton ATPase subunit D OS=Homo sapiens GN=ATP6V1D PE=1 SV=1	28,26	10,00	31,00	6,00	0,76	3,44	0,56	0,69
IDHP	Isocitrate dehydrogenase [NADP], mitochondrial OS=Homo sapiens GN=IDH2 PE=1 SV=2	50,91	9,00	18,00	9,00	0,49	5,11	0,60	0,75
TPD54	Tumor protein D54 OS=Homo sapiens GN=TPD52L2 PE=1 SV=2	22,24	9,00	23,00	4,00	2,19	0,50	0,63	0,65
ODPA	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial OS=Homo sapiens GN=PDH	43,30	9,00	14,00	8,00	0,58	0,50	0,49	0,46
TCPH	T-complex protein 1 subunit eta OS=Homo sapiens GN=CCT7 PE=1 SV=2	59,37	8,00	15,00	7,00	0,75	2,03	0,53	0,70
QCR2	Cytochrome b-c1 complex subunit 2, mitochondrial OS=Homo sapiens GN=UQCRC2 PE=1 SV=3	48,44	8,00	19,00	7,00	0,51	0,54	2,75	0,39
SRC8	Src substrate cortactin OS=Homo sapiens GN=CTTN PE=1 SV=2	61,59	8,00	13,00	6,00	0,39	0,79	0,54	0,77
CYTB	Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	11,14	8,00	67,00	7,00	0,57	22,08	0,32	0,16
TOM1	Target of Myb protein 1 OS=Homo sapiens GN=TOM1 PE=1 SV=2	53,82	8,00	7,00	5,00	0,63	0,44	0,57	1,37
SH3L1	SH3 domain-binding glutamic acid-rich-like protein OS=Homo sapiens GN=SH3BGRL PE=1 SV=1	12,77	7,00	18,00	5,00	0,70	9,38	0,63	0,42
ITB1	Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2	88,42	7,00	13,00	5,00	0,12	2,33	0,61	0,42
TB182	182 kDa tankyrase-1-binding protein OS=Homo sapiens GN=TNKS1BP1 PE=1 SV=4	181,80	7,00	3,00	3,00	0,19	0,09	1,94	0,59
CALX	Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2	67,57	6,00	24,00	8,00	0,60	2,91	0,69	0,69
HNRPD	Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens GN=HNRNPD PE=1 SV=1	38,43	6,00	7,00	5,00	0,59	0,75	0,47	0,53
BAP31	B-cell receptor-associated protein 31 OS=Homo sapiens GN=BCAP31 PE=1 SV=3	27,99	6,00	16,00	5,00	0,53	10,28	0,16	0,46
HNRPQ	Heterogeneous nuclear ribonucleoprotein Q OS=Homo sapiens GN=SYNCRIP PE=1 SV=2	69,60	6,00	18,00	7,00	0,64	0,86	0,42	0,56
ASC	Apoptosis-associated speck-like protein containing a CARD OS=Homo sapiens GN=PYCARD PE=1 SV=2	21,63	5,00	9,00	3,00	0,62	2,00	0,73	0,71
LGUL	Lactoylglutathione lyase OS=Homo sapiens GN=GLO1 PE=1 SV=4	20,78	5,00	11,00	5,00	0,61	2,58	0,64	0,58
E41L3	Band 4.1-like protein 3 OS=Homo sapiens GN=EPB41L3 PE=1 SV=2	120,68	5,00	3,00	3,00	0,67	0,80	0,28	0,75
SCAM2	Secretory carrier-associated membrane protein 2 OS=Homo sapiens GN=SCAMP2 PE=1 SV=2	36,65	5,00	9,00	3,00	0,80	0,55	0,53	0,07
RU17	U1 small nuclear ribonucleoprotein 70 kDa OS=Homo sapiens GN=SNRNP70 PE=1 SV=2	51,56	5,00	12,00	4,00	0,50	0,65	1,00	0,60
GLOD4	Glyoxalase domain-containing protein 4 OS=Homo sapiens GN=GLOD4 PE=1 SV=1	34,79	5,00	5,00	3,00	0,77	2,19	0,52	0,51
AP1B1	AP-1 complex subunit beta-1 OS=Homo sapiens GN=AP1B1 PE=1 SV=2	104,64	5,00	6,00	5,00	0,88	0,64	0,77	0,47
VPS4B	Vacuolar protein sorting-associated protein 4B OS=Homo sapiens GN=VPS4B PE=1 SV=2	49,30	4,00	7,00	4,00	0,61	0,68	1,29	0,59
SH3G1	Endophilin-A2 OS=Homo sapiens GN=SH3GL1 PE=1 SV=1	41,49	4,00	7,00	4,00	0,63	0,62	0,68	1,33
RAN	GTP-binding nuclear protein Ran OS=Homo sapiens GN=RAN PE=1 SV=3	24,42	4,00	11,00	6,00	0,43	2,49	0,43	0,59
CYB5	Cytochrome b5 OS=Homo sapiens GN=CYB5A PE=1 SV=2	15,33	4,00	5,00	3,00	0,69	0,22	0,63	0,48
PTN11	Tyrosine-protein phosphatase non-receptor type 11 OS=Homo sapiens GN=PTPN11 PE=1 SV=2	68,44	4,00	3,00	2,00	0,73	0,68	0,67	0,82
H2A1J	Histone H2A type 1-J OS=Homo sapiens GN=HIST1H2AJ PE=1 SV=3	13,94	4,00	6,00	2,00	0,37	0,47	2,05	0,20
MPRI	Cation-independent mannose-6-phosphate receptor OS=Homo sapiens GN=IGF2R PE=1 SV=3	274,38	4,00	4,00	3,00	0,52	0,66	0,87	0,70
RHOG	Rho-related GTP-binding protein RhoG OS=Homo sapiens GN=RHOG PE=1 SV=1	21,31	3,00	7,00	4,00	0,50	0,73	0,64	1,24
FPPS	Farnesyl pyrophosphate synthase OS=Homo sapiens GN=FDPS PE=1 SV=4	48,28	3,00	9,00	5,00	0,66	0,72	0,35	0,50
BIEA	Biliverdin reductase A OS=Homo sapiens GN=BLVRA PE=1 SV=2	33,43	3,00	7,00	3,00	1,28	0,64	0,72	0,52
AP1S2	AP-1 complex subunit sigma-2 OS=Homo sapiens GN=AP1S2 PE=1 SV=1	18,62	3,00	7,00	2,00	0,67	0,46	0,54	2,13
SRSF7	Serine/arginine-rich splicing factor 7 OS=Homo sapiens GN=SRSF7 PE=1 SV=1	27,37	3,00	11,00	5,00	1,10	0,65	0,61	0,58
IF4G2	Eukaryotic translation initiation factor 4 gamma 2 OS=Homo sapiens GN=EIF4G2 PE=1 SV=1	102,36	3,00	7,00	4,00	0,69	0,59	0,99	0,68
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate OS=Homo sapiens GN=HGS PE=1 SV=1	86,19	2,00	4,00	2,00	0,65	0,74	0,50	0,51
RL23A	60S ribosomal protein L23a OS=Homo sapiens GN=RPL23A PE=1 SV=1	17,70	2,00	12,00	4,00	0,58	0,77	0,44	0,39
H2B1L	Histone H2B type 1-L OS=Homo sapiens GN=HIST1H2BL PE=1 SV=3	13,95	2,00	12,00	3,00	1,26	0,69	0,76	0,72
VAPA	Vesicle-associated membrane protein-associated protein A OS=Homo sapiens GN=VAPA PE=1 SV=3	27,89	2,00	5,00	3,00	0,49	0,70	0,91	0,74
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1 OS=Homo sapiens GN=AHSA1 PE=1 SV=1	38,27	1,00	6,00	3,00	1,01	0,31	0,26	0,24
RS30	40S ribosomal protein S30 OS=Homo sapiens GN=FAU PE=1 SV=1	6,65	1,00	6,00	3,00	0,12	0,33	1,75	0,28
AT1A1	Sodium/potassium-transporting ATPase subunit alpha-1 OS=Homo sapiens GN=ATP1A1 PE=1 SV=1	112,90	24,00	70,00	18,00	1,71	2,54	1,85	1,84
TBB6	Tubulin beta-6 chain OS=Homo sapiens GN=TUBB6 PE=1 SV=1	49,86	15,00	41,00	7,00	1,77	1,74	0,86	1,58
QCR1	Cytochrome b-c1 complex subunit 1, mitochondrial OS=Homo sapiens GN=UQCRC1 PE=1 SV=3	52,65	12,00	25,00	8,00	1,58	1,66	0,72	1,49
RS20	40S ribosomal protein S20 OS=Homo sapiens GN=RPS20 PE=1 SV=1	13,37	4,00	12,00	5,00	2,38	0,34	2,09	2,17
LIRB4	Leukocyte immunoglobulin-like receptor subfamily B member 4 OS=Homo sapiens GN=LILRB4 PE=1 SV=3	49,36	3,00	10,00	5,00	1,72	1,50	1,25	1,32
TIF1B	Transcription intermediary factor 1-beta OS=Homo sapiens GN=TRIM28 PE=1 SV=5	88,55	3,00	6,00	4,00	1,56	0,63	1,64	1,56
PGAM5	Serine/threonine-protein phosphatase PGAM5, mitochondrial OS=Homo sapiens GN=PGAM5 PE=1 SV=2	32,00	1,00	5,00	4,00	1,53	1,38	0,24	1,71

Chapter summary

Our main achievements (**Research Article 2**) are summarized as followed:

- Gel-based approach demonstrated that macrophages exposed to 5 cumulative ionizing radiation doses up to 10 Gy exhibit expression alterations in 17 proteins, being 10 downregulated and 7 upregulated. GO analysis indicates that these proteins are significantly associated with *regulation of biological quality*, *catabolic processes*, and *response to stimulus* (biological processes). Regarding molecular function, most of the identified proteins present predicted *catalytic activity*, are mainly located in the cytoplasm, either in *mitochondria, cytosol* or even associated with *cytoplasmic vesicles*. A positive association with *aerobic respiration*, *regulation of transcription factor import into nucleus namely NF-κB* and *ATP biosynthetic process* was also found;

- Gel-free approach demonstrated that macrophages exposed to 5 cumulative ionizing radiation doses up to 10 Gy exhibit expression alterations in 67 proteins, being 60 downregulated and 7 upregulated. The GO analysis indicated that these proteins are significantly associated with the following biological processes: *regulation of viral release from host cell*, *protein localization to endoplasmic reticulum*, *regulation of exosomal secretion*, *cellular aldehyde metabolic process*, *monosaccharide metabolic process*, *post-Golgi vesicle-mediated transport* and *aerobic respiration*. Some of them are suggested to be downregulated as: *protein folding in endoplasmic reticulum*, *antigen processing and presentation of peptide antigen via MHC class II, maintenance of protein location in cell, regulation of cell shape, cellular response to alcohol, negative regulation of cellular amide metabolic process, regulation of mRNA metabolic process* and *cellular aldehyde metabolic process*;

- Despite DNA damage, irradiated macrophages remain metabolically viable, and reduce both glucose uptake and total cellular ATP levels;

- A conjugation of both gel-based and gel-free approaches revealed that irradiated macrophages present the downregulation of cathepsin D, an enzyme associated with antigen processing/presentation and apoptosis regulation, and the upregulation of transferrin receptor (CD71), a mediator of iron uptake, as further validated by western blot analysis.

Chapter V

Research article 3

Intricate macrophage-colorectal cancer cell communication in response to radiation

|Manuscript conditionally accepted at *PLOS ONE*

Chapter overview

After characterizing the macrophage response to radiation, from a functional (Chapter III) and signalling (Chapter IV) point of views, we found imperative to understand how the communication between macrophages and cancer cells was affected by ionizing radiation. Thus, the present chapter contains experimental results addressing the second aim of this PhD thesis - *To reveal how ionizing radiation exposure affects macrophage-cancer cell communication*. Macrophages and colorectal cancer cells, irradiated with 5 cumulative ionizing radiation doses (2 Gy/fraction/day), alone or in co-culture, were used as working models. Cancer cell response to radiation was characterized through evaluation of apoptosis induction and expression of metabolism- and survival-related genes. Additionally, macrophage polarization status upon co-culture with cancer cells, with or without radiation exposure, was also evaluated. The effect of irradiated co-cultures on the activity of non-irradiated cancer cells was also addressed.

Data is mainly compiled in an original research manuscript with the respective supplementary information, which is conditionally accepted at *PLOS ONE*. At the end of this chapter, a brief report of complementary, but not yet published results, is also presented. It aims to explore i) the macrophage-cancer cell co-culture upon irradiation, beyond results presented in the manuscript, as well as ii) the effect of irradiated macrophages on non-irradiated cells through released signals.

Research Article 3

Intricate macrophage-colorectal cancer cell communication in response to radiation

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Abstract

Both cancer and tumour-associated host cells are exposed to ionizing radiation when a tumour is subjected to radiotherapy. Macrophages frequently constitute the most abundant tumour-associated immune population, playing a role in tumour progression and response to therapy. The present work aimed to evaluate the importance of macrophage-cancer cell communication in the cellular response to radiation.

To address this question, we established monocultures and indirect co-cultures of human monocyte-derived macrophages with RKO or SW1463 colorectal cancer cells, which exhibit higher and lower radiation sensitivity, respectively. Mono- and co-cultures were then irradiated with 5 cumulative doses, in a similar fractionated scheme to that used during cancer patients' treatment (2 Gy/fraction/day).

Our results demonstrated that macrophages sensitize RKO to radiation-induced apoptosis, while protecting SW1463 cells. Additionally, the co-culture with macrophages increased the mRNA expression of metabolism- and survival-related genes more in SW1463 than in RKO. The presence of macrophages also upregulated glucose transporter 1 expression in irradiated SW1463, but not in RKO cells. In addition, the influence of cancer cells on the expression of pro- and anti-inflammatory macrophage markers, upon ionizing radiation exposure, was also evaluated. Our data demonstrated that the response of both macrophages and cancer cells to radiation may be mutually influenced. Notably, conditioned medium from irradiated co-cultures increased non-irradiated RKO cell migration and invasion.

Overall, the establishment of primary human macrophage-cancer cell co-cultures revealed an intricate cell communication in response to ionizing radiation, which should be considered when developing therapies adjuvant to radiotherapy.

Keywords: Tumour microenvironment; Human monocyte-derived macrophages; Colorectal cancer cells; Radioresistance/Radiosensitivity; Ionizing radiation; Co-culture

Introduction

Tumours are complex ecosystems involving much more than solely cancer cells. They are characterized by a dynamic tumour microenvironment supported by extracellular matrix components and several tumour-associated cells, which altogether modulate cancer cell activities, dictating the success of tumour progression[1,2]. Amongst tumour-associated cells, macrophages are particularly relevant, as they constitute, in many solid tumours, the most abundant immune population, and are known as obligate partners for cancer cell migration, invasion and metastasis[3,4].

Macrophages not only contribute to tumour progression, as they may also modulate tumour response to therapy[5,6], particularly to radiotherapy, one of the most common anti-cancer treatments, being employed in approximately 50% of all cancer patients at some point of their treatment[7]. Radiotherapy is typically delivered as a multi-fractionated rather than single-dose regimen, involving daily doses of 2 Gy (5 fractions/week), during several weeks of treatment[8]. In animal models, the depletion of tumour-associated macrophages, either local or systemically, prior radiotherapy, decreases tumour regrowth, favouring the anti-tumour effects of ionizing radiation[9]. Contrarily, co-implantation of tumour cells with bone marrow–derived macrophages increases tumour radioresistance[9], although macrophages are also able to radiosensitize tumour cells, for instance through the induction of NO synthesis[10].

In disease as well as in homeostasis, macrophages exhibit a functional phenotype that may vary between two extremes of a continuous spectrum of activation[11]. Pro-inflammatory macrophages are characterized by the production of high levels of pro-inflammatory mediators, such as TNF-α, IL1-β, IL-6 or IL-12 and are associated with bacterial clearance and tumour cytotoxicity, being considered tumour suppressors[11]. On its turn, anti-inflammatory macrophages are high producers of anti-inflammatory mediators, such as IL-10 or TGF-β, and are mainly involved in extracellular matrix remodelling and immune suppression, being considered tumour promoters[11]. In tumours, macrophages frequently acquire an anti-inflammatory profile[12] and their modulation towards a pro-inflammatory phenotype has been pointed as another possible strategy to modulate tumour cell response to therapy[6, 13].

Although macrophages may play a role in tumour cell radioresistance, this may also be intrinsically determined, namely by *p53* mutations[14,15] and chromosomal instability in tumour cells[16]. Additionally, alterations in DNA repair efficiency[17], upregulation of the pro-survival protein Bcl-xL[18], enhanced aerobic glycolysis[14], and altered mitochondrial function[19] may also contribute to acquired resistance to radiation-induced apoptosis. Overall, cancer cell response to radiation has

been intensively investigated and it is now well-characterized[20]. Nonetheless, only more recently, attention has been paid to the effect of radiation on tumour-associated host cells, as they were found to play a role in radiotherapy outcome[21,22]. We have recently provided new insights into this field, by exploring the effect of clinically relevant ionizing radiation doses on macrophage function and survival[23].

To design effective therapies to radiosensitize cancer cells in the presence of macrophages, it is crucial to understand how macrophage and cancer cell communication affects the response to radiation. To address this issue, we established indirect macrophage-cancer cell co-cultures and exposed the whole system to cumulative ionizing radiation doses, in a fractionated scheme similar to the one used for cancer patients' treatment (2 Gy/fraction/day). We selected two colorectal cancer cell lines, RKO and SW1463, known to exhibit, respectively, a radiation sensitive[24] and a radiation resistant profile[25], and co-cultured them with human monocyte-derived macrophages. This *in vitro* approach constitutes the basis of the present study and allowed us to characterize two important hallmarks of cancer cells, the resistance to cell death and the deregulation of cellular energetics, and to profile the macrophage activation state upon co-culture irradiation. Additionally, the role of irradiated co-culture conditioned medium on other hallmarks of cancer, namely cell invasion, migration and angiogenesis was also addressed.

Results

To address the proposed goals, an indirect co-culture system was established, which involved the crosstalk of human monocyte-derived macrophages with two colorectal cancer cell lines, RKO or SW1463, known to exhibit high and low sensitivity to radiation, respectively[24,25]. Monocultures and co-cultures were then subjected to cumulative ionizing radiation doses for 5 days, using a fractionated scheme similar to the one employed during cancer patients' treatment (2 Gy/fraction/day) (Figure 1).

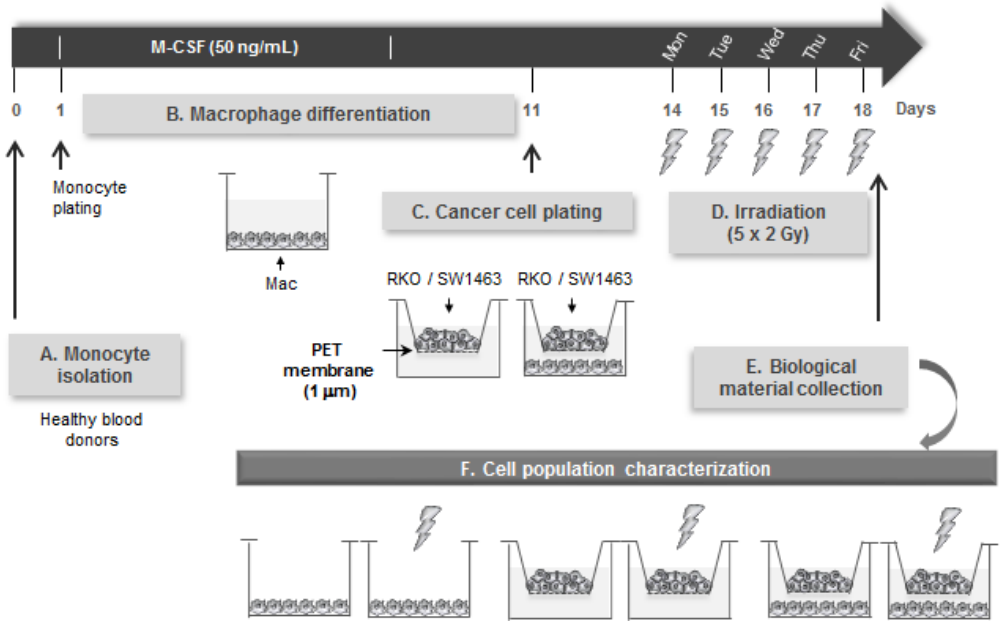

Figure 1 - Schematic overview of the methodology used in this work. Monocytes were isolated from the peripheral blood of healthy blood donors, and cultured, for seven days, with M-CSF to allow their differentiation into macrophages. On day 11, RKO or SW1463 cancer cells were cultured in transwell inserts of 1 µm pore size, on top of macrophages, and the whole set was then irradiated with 2 Gy/fraction/day, for 5 days. Conditioned medium (CM) of irradiated co-cultures, as well as protein and RNA from individual cell populations, were collected 6 h after the last ionizing radiation dose, and compared with the respective controls.

The presence of macrophages reduces radiation-induced apoptosis in SW1463, but not in RKO cells

Since ionizing radiation triggers a cascade of molecular events that ultimately leads to apoptosis, we evaluated the activation of two major proteins of the apoptotic signalling cascade, caspase-3 and poly (ADP-ribose) polymerase (PARP). Our results demonstrated that ionizing radiation enhanced cleaved PARP and cleaved caspase-3 levels in both RKO and SW1463 cells, either cultured alone or in combination with macrophages (Figure 2). Interestingly, in comparison with mono-cultures, the presence of macrophages enhanced both PARP and caspase-3 cleavage in RKO cells, while reduced both proteins cleavage in SW1463 cells, upon radiation exposure (Figure 2).

Notably, the presence of macrophages *per se* (without radiation exposure) decreased cleaved PARP and cleaved caspase-3 expression levels in SW1463 cells, while an increase was verified for RKO cells. These results suggest that macrophages may direct cancer cell response to ionizing radiation, particularly by sensitizing RKO cells and promoting the radioresistance of SW1463 cells to radiation-induced cell death.

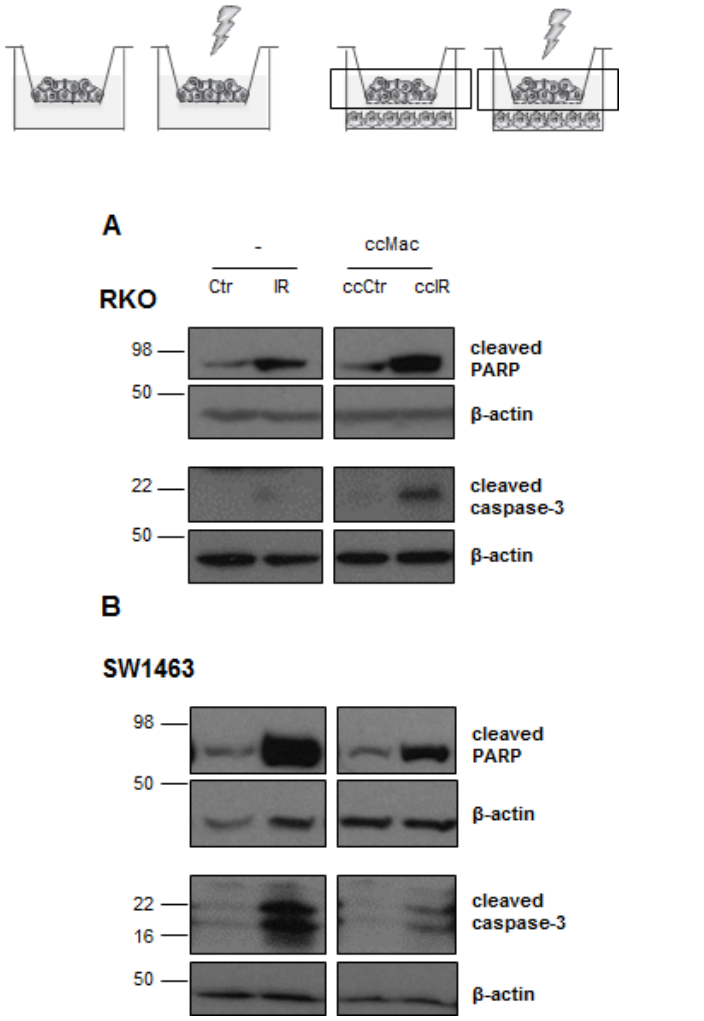

Figure 2 - Expression of both PARP and caspase-3 cleavage in RKO and SW1463 cancer cells. A) RKO and **B)** SW1463 cancer cells were cultured alone (-) or in the presence of macrophages (ccMac), with (IR or ccIR, 5 x 2 Gy) or without (Ctr or ccCtr) radiation exposure. Both cleaved PARP and cleaved caspase-3 expression levels were evaluated 6 h after irradiation by western blot analysis.

In order to understand SW1463 cancer cell enhanced radioresistance in the presence of macrophages, we first evaluated whether it could be attributed to a reduction of ionizing radiation-induced DNA damage signalling. Therefore, the expression and phosphorylation status of the Checkpoint kinase 2 (Chk2), a protein involved in the propagation of DNA damage signals[27], were evaluated 6 h after irradiation (Supplementary Figure S1). Our results demonstrated that Chk2 phosphorylation increased in SW1463 irradiated alone or in co-culture, when compared to the respective non-irradiated controls, indicating, as expected, higher DNA damage levels in irradiated cells. However, no major alterations were found between Chk2 phosphorylation levels in SW1463 cells cultured alone or in combination with macrophages, suggesting that macrophages may increase SW1463 radioresistance through alternative mechanisms rather than DNA damage reduction.

Increased expression of metabolism-related genes upon co-culture with macrophages may explain the reduced SW1463 apoptotic signalling after co-culture irradiation

In order to search for possible mechanistic explanations, we focused on two additional targets involved in anti-apoptotic response, Bcl-xL and Mcl-1, encoded by *BCL2L1* and *MCL1*, respectively (Figure 3A).

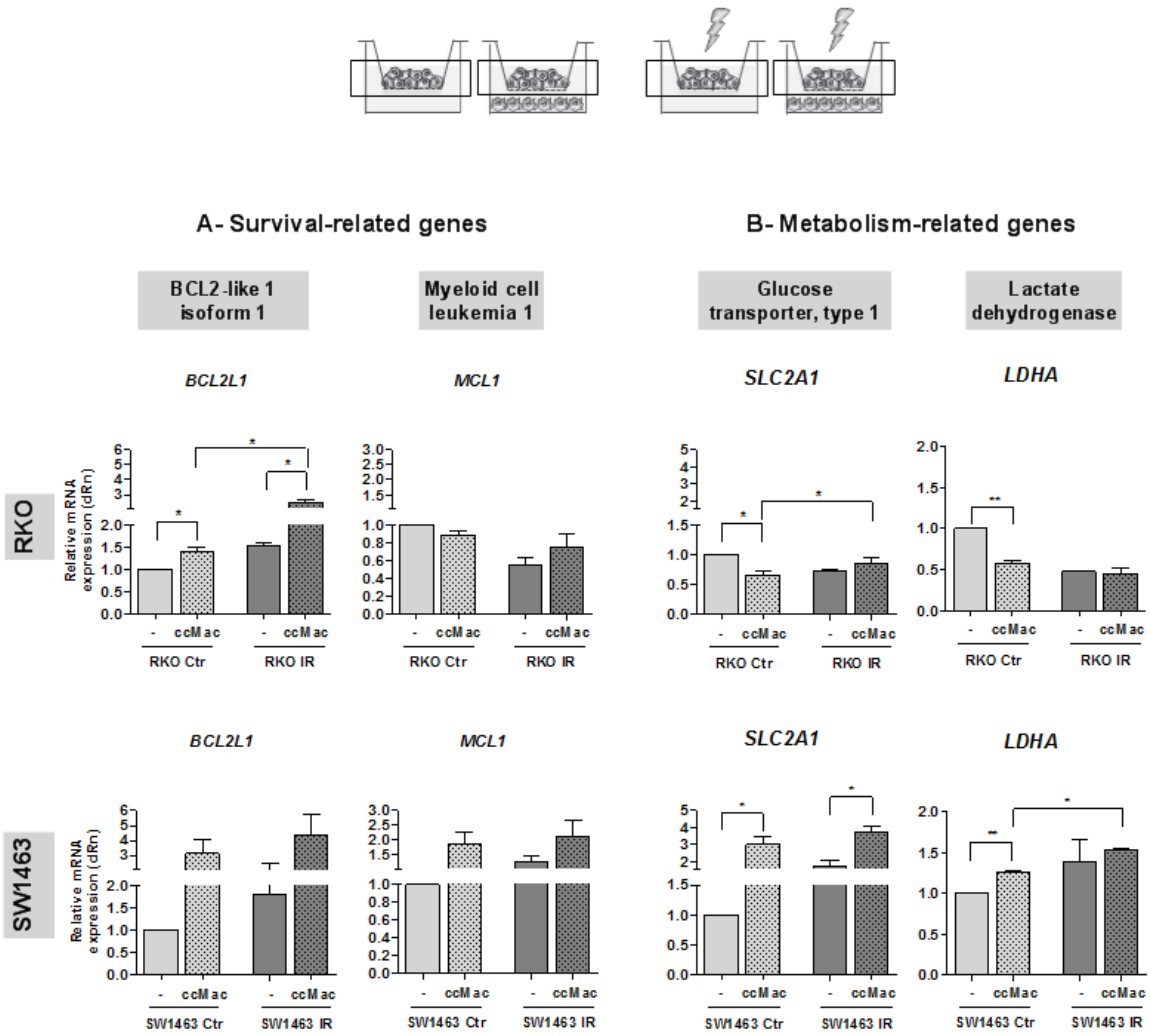

Figure 3 - Expression of survival- and metabolism-related targets in RKO and SW1463 cancer cells. Both RKO and SW1463 cancer cells were cultured alone (-) or in the presence of macrophages (ccMac), with (IR, 5 x 2 Gy) or without (Ctr) radiation exposure. The mRNA expression levels of **A)** two survival-related genes, *BCL2L1* and *MCL1,* which encode the anti-apoptotic proteins Bcl-xL and Mcl-1, respectively, and **B)** of two metabolism-related genes, *SLC2A1* and *LDHA,* which encode the glucose transporter type 1 and the lactate dehydrogenase, respectively, were evaluated in cancer cells, 6 h after irradiation. Graphs result from the relative mRNA quantification in cancer cells cultured with macrophages from distinct donors (n = 4 per each cell line), evaluated in four independent experiments. * $P < 0.05$, ** $P < 0.01$.

Results demonstrate that upon co-culture with macrophages, RKO cells increased *BCL2L1* expression, without alterations in *MCL1*, independently of ionizing radiation treatment. In SW1463 cells, macrophages tend to increase the expression levels of both *BCL2L1* and *MCL1* upon irradiation, although without achieving statistical significance.

Since mitochondrial dysfunction after radiation exposure may compromise energy supply[28] and cell function[29], we focused on the expression of two metabolism-related genes, *SLC2A1* and *LDHA* (Figure 3B), which frequent overexpression by cancer cells contribute to increased glycolysis[30]. *SLC2A1* encodes the glucose transporter 1 (GLUT1), a membrane-bound protein responsible for glucose uptake, while *LDHA* encodes the lactate dehydrogenase A, an enzyme responsible for the conversion of pyruvate to lactate. Notably, macrophages decreased RKO cells *SLC2A1* and *LDHA* expression, but only in the absence of ionizing radiation. Contrarily, macrophages enhanced SW1463 *SLC2A1* expression in both irradiated and non-irradiated conditions and *LDHA* without radiation exposure. Additionally, an increase of *LDHA* expression in irradiated versus non-irradiated SW1463 co-cultures, but not in RKO co-cultures, was found, suggesting that SW1463 cells exhibit an adaptive metabolic response and a more radiation resistant profile, as indicated by the literature[25].

Overall, our results revealed that for RKO cells, macrophages overall effect leads to enhanced apoptosis, despite the increased mRNA expression of the pro-survival *BCL2L1*. For SW1463, macrophages contribute to decrease apoptosis, which may be achieved through metabolic alterations induced by enhanced glucose transporter expression.

Colorectal cancer cells promote a macrophage pro-inflammatory phenotype, with or without radiation exposure

Besides interfering with survival- and metabolism-related targets in cancer cells, the presence of macrophages also modulated the expression of macrophage-stimulating factor 1 (*CSF1*), an important molecule for macrophage recruitment. Notably, in response to radiation, the presence of macrophages induced upregulation of *CSF1* in RKO, but not in SW1463 cells (Supplementary Figure S2). This result led us to investigate whether RKO and SW1463 cancer cells could differently modulate macrophage response to radiation. To address this question, we compared the inflammatory profile of macrophages cultured in the presence of each of the colorectal cancer cells with the one of macrophages monocultures, 6 h after radiation exposure (third versus fourth column of each graph, Figures 4 and 5). The macrophage inflammatory profile was assessed by mRNA expression analysis of a panel of pro-inflammatory (*CD80, CCL2, CXCL8, TNF*, *CCR7, IL6*, *IL1B* and *CCL5*) and anti-inflammatory (*CD163*, *IL10*, *CCL18* and *VCAN*) genes.

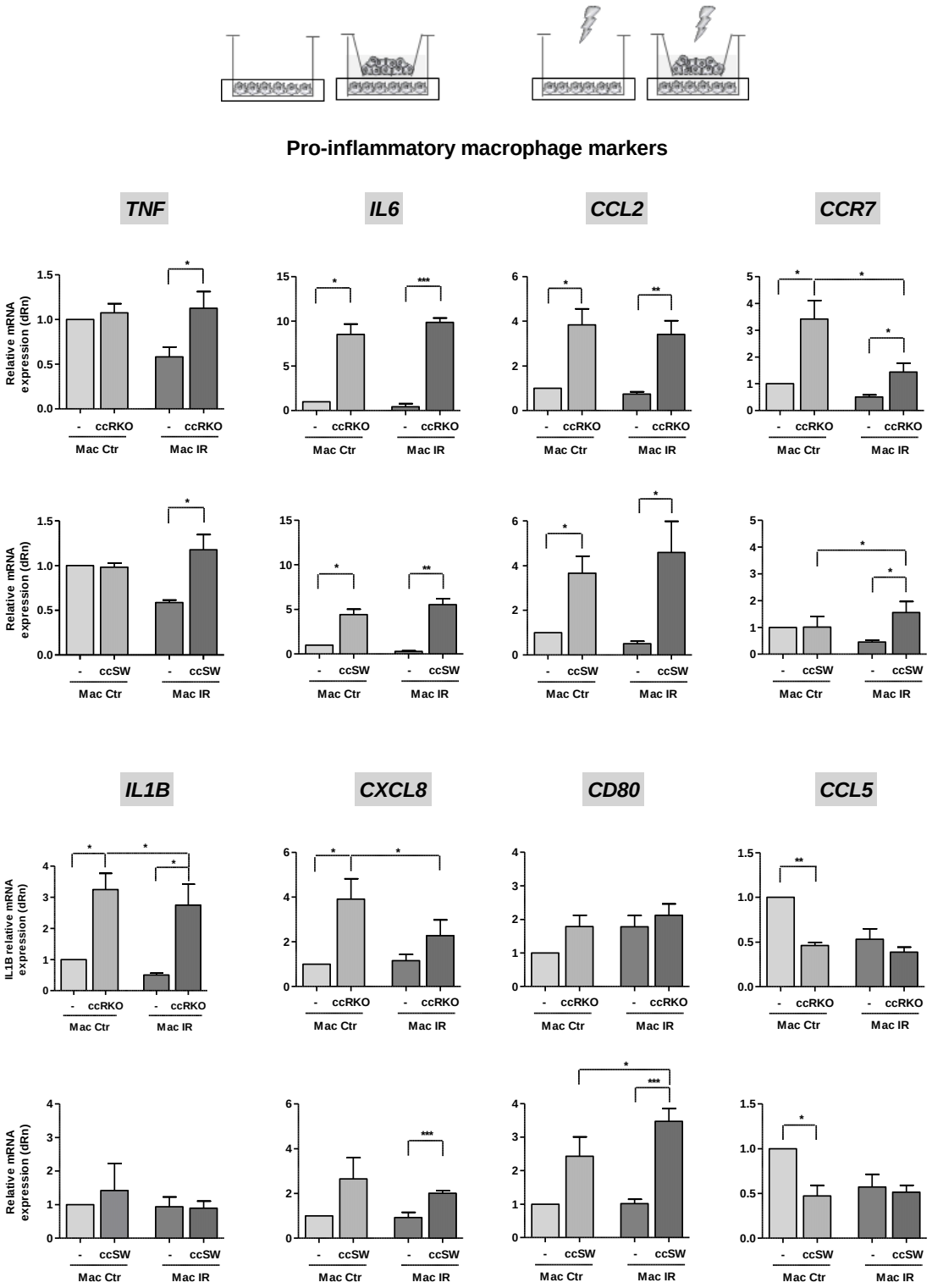

Figure 4 - The mRNA expression of pro-inflammatory markers in irradiated macrophages, cultured with RKO or SW1463 cells. Macrophages were cultured alone (-) or in the presence of RKO or SW1463 cancer cells (ccRKO or ccSW), with (IR, 5 x 2 Gy) or without (Ctr) radiation exposure. The mRNA expression of a panel of pro-inflammatory macrophage markers (*TNF*, *IL6*, *CCL2*, CCR7, *IL1B*, *CXCL8*, *CD80* and *CCL5*) was evaluated 6 h after irradiation. Graphs result from the relative mRNA quantification in macrophages cultured with RKO or SW1463 (*n* = 4 per each cell line), evaluated in four independent experiments. For simplicity, SW1463 cells were indicated as "SW". * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

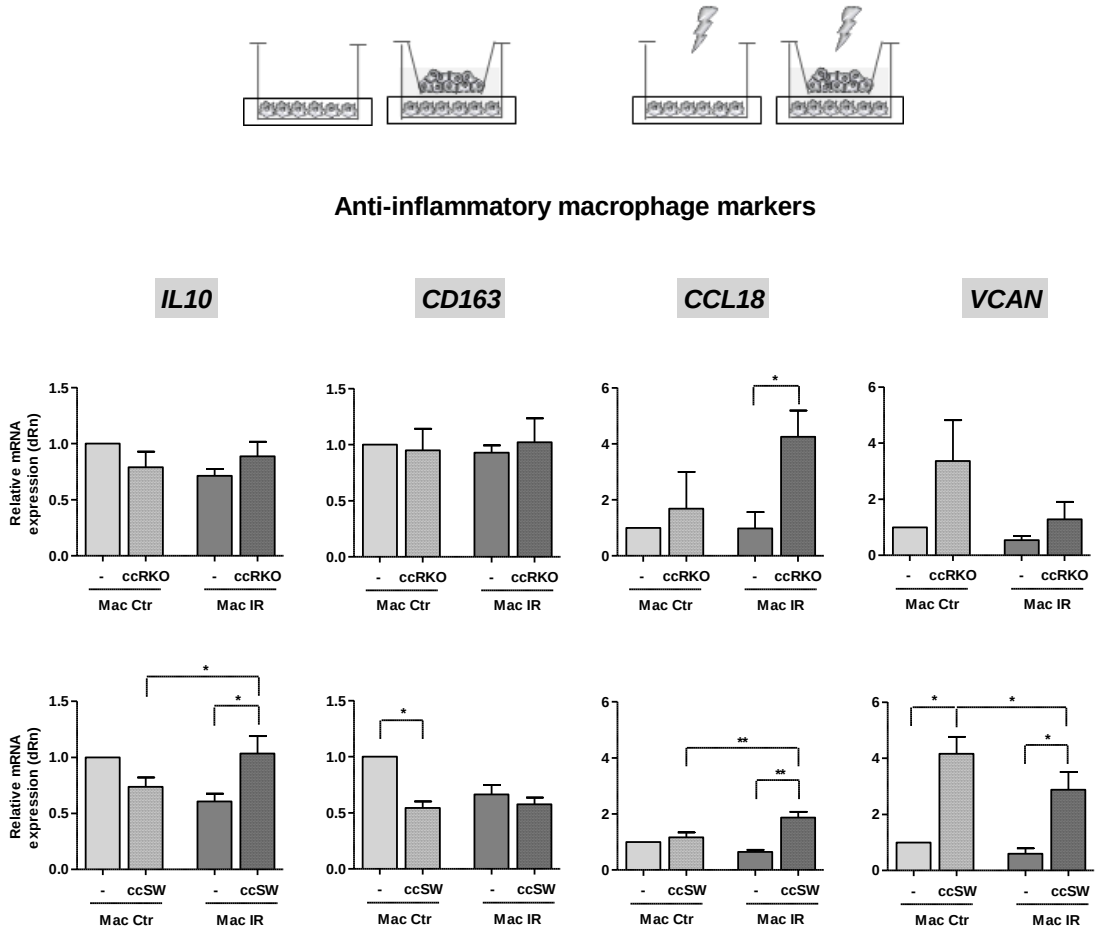

Figure 5 - The mRNA expression of anti-inflammatory markers in irradiated macrophages, cultured with RKO or SW1463 cells. Macrophages were cultured alone (-) or in the presence of RKO or SW1463 cancer cells (ccRKO or ccSW), with (IR, 5 x 2 Gy) or without (Ctr) radiation exposure. The mRNA expression of a panel of anti-inflammatory macrophage markers (*IL10*, *CD163*, *CCL18* and *VCAN*) was evaluated 6 h after irradiation. Graphs result from the relative mRNA quantification in macrophages cultured with RKO or SW1463 (*n* = 4 per each cell line), evaluated in four independent experiments. For simplicity, SW1463 cells were indicated as "SW". * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Our results demonstrated that in the presence of ionizing radiation either RKO or SW1463 cancer cells increased mRNA expression levels of the macrophage pro-inflammatory markers *TNF, IL6*, *CCL2 and CCR7* (Figure 4) as well as of the anti-inflammatory marker *CCL18* (Figure 5). Despite these molecular similarities, RKO, but not SW1463 cells, enhanced the expression of the macrophage pro-inflammatory marker *IL1B*. Additionally, SW1463, but not RKO cells, increased the expression of *CXCL8* and *CD80* pro-inflammatory (Figure 4), and of *VCAN* and *IL10* anti-inflammatory markers (Figure 5).

Particularly, we observed that, in the absence of ionizing radiation both colorectal cancer cells had already the ability to modulate macrophage inflammatory profile (first versus second column of each graph, Figures 4 and 5). Consistently, in the presence of either RKO or SW1463 cells, macrophages exhibited increased expression of *IL6 and CCL2,* while decreased the expression of *CCL5* pro-inflammatory markers. Although *CXCL8* and *VCAN* tend to increase in macrophages cultured with both cancer cells, statistical significance was only achieved for the anti-inflammatory marker *VCAN* in co-cultures with SW1463 and for the pro-inflammatory marker *CXCL8* marker in co-cultures with RKO cells. In addition, RKO also induced a significant increase of *IL1B* and *CCR7* pro-inflammatory markers, while SW1463 cells significantly reduced the expression of the anti-inflammatory *CD163* receptor. Altogether, these data suggest that both RKO and SW1463 colorectal cancer cells promoted, even in the absence of ionizing radiation, a macrophage pro-inflammatory phenotype.

To distinguish the molecular alterations dependent on irradiation from those induced by the presence of colorectal cancer cells, the mRNA expression levels of macrophages from irradiated co-cultures were compared with those from non-irradiated co-cultures (second versus fourth column of each graph, Figures 4 and 5). Most strikingly, our results evidenced that ionizing radiation significantly reduced the expression of *CCR7* pro-inflammatory receptor on macrophages co-cultured with RKO, while enhanced it on macrophages co-cultured with SW1463 cells (Figure 4). Additionally, for co-cultures established in the presence of RKO cells, radiation also decreased significantly the expression of the pro-inflammatory chemokine *CXCL8* (Figure 4). However, for co-cultures established in the presence of SW1463 cells, ionizing radiation increased significantly the expression of the pro-inflammatory *CD80* receptor and of the *CCL18* cytokine (Figure 4) and of the anti-inflammatory *IL10* cytokine, while reduced the expression of the anti-inflammatory extracellular matrix *VCAN* (Figure 5). No major alterations were found between the levels of the pro-inflammatory *TNF, IL6*, *CCL2* and *CCL5* and of the anti-inflammatory *CD163* in macrophages cultured in the presence of cancer cells, with or without radiation exposure (Figures 4 and 5).

Conditioned medium from irradiated macrophage-RKO co-culture increased migration and invasion of non-irradiated RKO cells

To evaluate how irradiated co-cultures affected the activity of non-irradiated cells, the ability of conditioned medium (CM) from irradiated and non-irradiated co-cultures to interfere with some hallmarks of cancer, namely invasion, migration and angiogenesis, was investigated (Figure 6). We have previously described that invasion, the hallmark of cancer that involves the ability of cancer

cells to cross the basement membrane and migrate through the nearby tissues, is also mediated by factors released by tumour-associated cells, as macrophages[1, 26]. Considering this, the invasion ability of non-irradiated cancer cells was evaluated in the presence of CM from either irradiated or non-irradiated co-cultures, using transwell inserts with a porous membrane coated with Matrigel, mimicking the basement membrane matrix (Figure 6A). Our results revealed that CM from irradiated co-cultures significantly increased the invasion of non-irradiated RKO ($P < 0.05$), in comparison with CM from non-irradiated co-cultures. However, no major alterations were observed in the invasion ability of SW1463 cells stimulated with CM from irradiated and non-irradiated co-cultures. For the analysis of cancer cell migration, non-irradiated RKO or SW1463 cancer cells were grown until confluence in a two chamber well and then stimulated with CM from irradiated or non-irradiated macrophages-cancer cell co-cultures (Figure 6B). After wound formation, RKO or SW1463 were able to migrate from both chambers towards the centre. Results demonstrated that after 48 h, CM from irradiated co-cultures stimulated RKO migration ($P < 0.05$), filling the empty area of the wound more efficiently than RKO stimulated with CM from non-irradiated co-cultures. However, no migration ability was observed for SW1463 in the presence of CM of either irradiated or non-irradiated co-cultures.

Since macrophages and cancer cells are able to induce angiogenesis through the secretion of several pro-angiogenic growth factors and cytokines/chemokines[1], the angiogenic potential of CM from irradiated and non-irradiated co-cultures was evaluated using the chick chorioallantoic membrane (CAM) model (Figure 6C). Notably, CM from irradiated co-cultures of macrophages with either RKO or SW1463 cancer cells had a similar angiogenic response as CM from non-irradiated co-cultures.

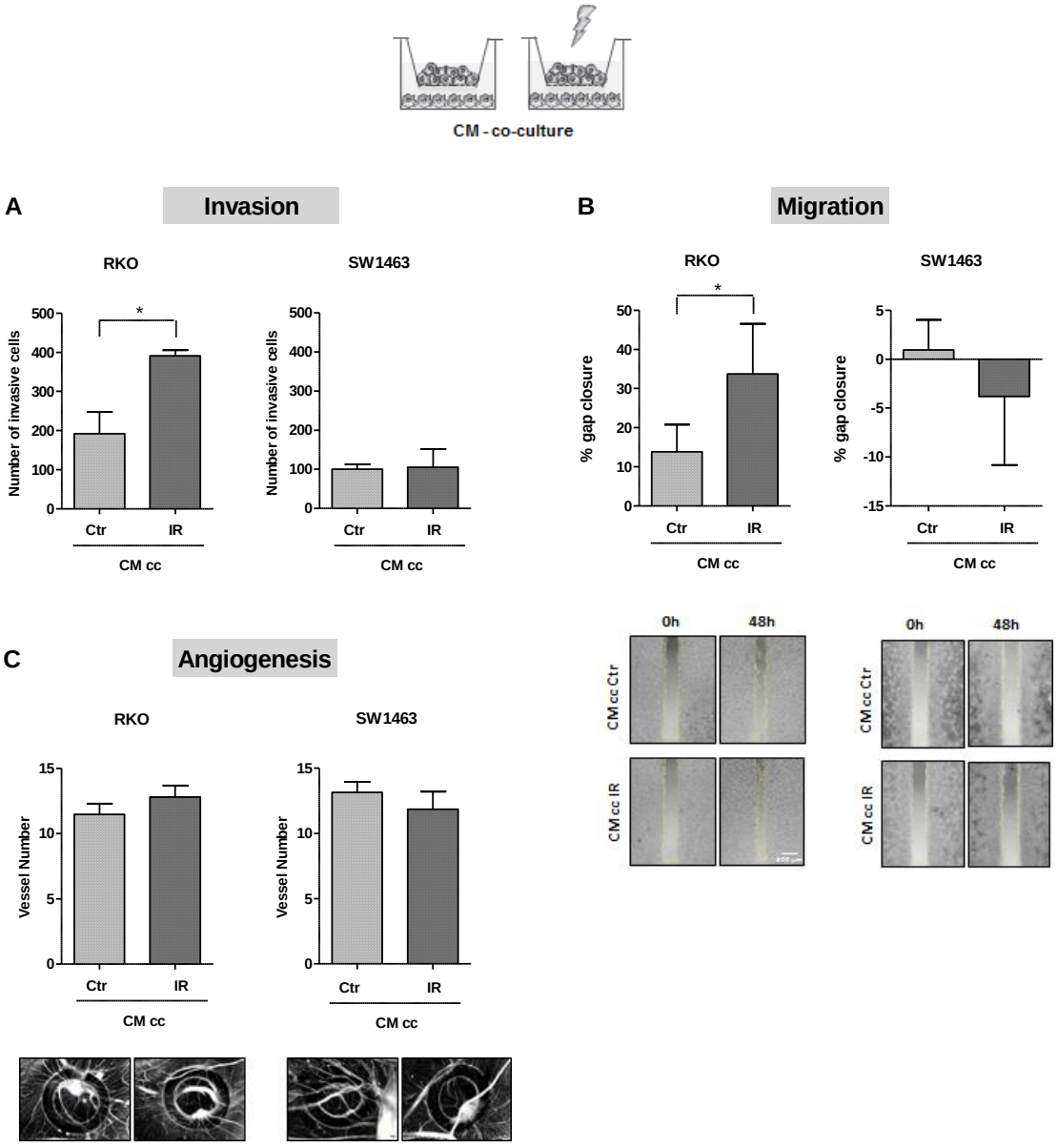

Figure 6 - Effect of conditioned medium from irradiated co-cultures in the invasion, migration and angiogenesis of non-irradiated cells. The ability of conditioned medium (CM), from irradiated (IR, 5 x 2 Gy) and non-irradiated (Ctr) co-cultures (cc) of macrophages with RKO or SW1463, to promote cancer cell migration and invasion (*n* = 4 per each cell line), as well as to induce angiogenesis, was evaluated. **A)** Non-irradiated RKO and SW1463 cells were seeded on Matrigel-based transwells and stimulated with CM from irradiated and non-irradiated co-cultures. After 24 h, the number of invasive cells was counted. **B)** Both non-irradiated RKO and SW1463 were plated until confluence on both chambers of Ibidi culture-inserts for migration assay. After wound formation, cancer cells were stimulated with CM from irradiated and non-irradiated co-cultures and migrated area was quantified upon 48 h. **C)** Concentrated CM from irradiated or non-irradiated co-cultures was inoculated in rings, on the top of the chick embryo chorioallantoic membrane (CAM), for 72 h. Analysis of CM-induced angiogenesis was performed through quantification of the number of new vessels in control and experimental conditions.

Discussion

Our results demonstrated that macrophages enhance the cancer cell intrinsic response to radiation, this is, they promote RKO radiation sensitivity, while enhance SW1463 cells radioresistance. This effect could be attributed either to a different ability of the distinct cell lines to modulate macrophage phenotype, to a differential response to the presence of macrophages upon irradiation, or most probably, to a combination of both.

The communication of macrophages with cancer cells, and how both populations influence each other, has been explored by several studies, at cellular and molecular levels. Most of those *in vitro* studies rely on two main strategies, direct[31,32] or indirect[33-35] macrophage-cancer cell co-cultures or, alternatively, stimulation of one population with the supernatant of the other[32,36,37]. The preferential methodological approach seems to be indirect macrophage-cancer cell co-cultures, as this constitutes a simple and practical *in vitro* model. These studies also frequently use macrophage-like cells differentiated from THP-1 human monocytes[33,35,36], upon stimulation with phorbol myristate acetate (PMA), although these are not completely representative of primary human macrophages[38]. Although the knowledge about macrophage-cancer cell communication has improved in the last years, there is scarce information on how ionizing radiation may modulate macrophage inflammatory profile and how the molecular crosstalk established between macrophages and cancer cells affects the radiation response of both populations.

To address these questions, we established indirect co-cultures between primary human macrophage cultures and colorectal cancer cells (CRCs), using two cell lines with different radiation sensitivities[24,25]. Briefly, radiosensitive RKO cells express mutated *ATM*[39], similarly to patients with a genetic disorder characterized by hypersensitivity to ionizing radiation[40]. On the other hand, SW1463 cells express mutated *TP53* and *KRAS*[39], which may delay apoptosis[41], enhance repair of DNA double-strand breaks[42] or induce metabolic reprogramming[42,43], as a way of protecting cells from radiation-induced damage.

Besides mutations, that intrinsically determine cancer cell response to radiation, other events, with which macrophages may interfere, can also modulate it. Our data demonstrated that in the presence of macrophages, radioresistant SW1463 cells increased *SLC2A1*, *MCL1*, and *BCL2L1* to levels very similar to those exhibited without irradiation, suggesting that the presence of macrophages *per se* may induce some protection against ionizing radiation. In general, cancer cells with acquired radioresistance exhibit higher expression of the glucose transporter GLUT-1 (encoded by *SLC2A1*) and enhanced lactate production levels than parental cells, which are maintained or increased upon radiation exposure[44]. Accordingly, some authors reported that

although transient, the higher glucose uptake seems to occur concomitantly with increased *SLC2A1* mRNA levels[45]. Additionally, upregulation of *MCL1* mRNA expression upon ionizing radiation exposure was reported as an early response to cytotoxic stress, reaching its peak at 4 h after a single 20 Gy dose, backing to basal levels 24 h after irradiation[46]. It was hypothesized that the maintenance of high *MCL1* levels or their increase upon irradiation, associated with enhanced short-term cell viability, could allow repair of radiation-induced DNA damage and suppression of apoptotic response, even in the presence of high levels of active caspase-3[46-48]. Accordingly, Mcl-1 targeting or depletion may sensitize cancer cells to radiation-induced apoptosis[49]. Overall, it seems that Mcl-1 acts as an integrative signal, mediating the opposite actions of pro-survival and pro-apoptotic signalling[50], thereby being an attractive target for modulation of cell radioresistance. On its turn, Bcl-xL targeting was reported to reduce CRCs survival upon ionizing radiation exposure by highly increasing their apoptotic rate[51]. In addition to macrophage induced alterations, in cancer cell´s metabolic and pro-survival targets, the modulation of macrophage polarization profile by cancer cells may also help to explain why both RKO and SW1463 cancer cell lines responded differently to radiation in the presence of macrophages. In that respect, the increased expression, in irradiated macrophages upon co-culture with RKO cells, of the pro-inflammatory *IL1B*, a crucial molecule for macrophage-mediated tumouricidal activity[52], may support the macrophage-increased RKO radiosensitivity. On the other hand, increased levels of anti-inflammatory *IL10* in irradiated macrophages upon co-culture with SW1463 cells may support survival and consequent macrophage-promoted SW1463 radioresistance, since high *IL10* levels in tumour-associated macrophages may play a role in cancer progression[53] and resistance to therapy[54]. Additionally, we may also speculate that the simultaneous increase, in irradiated macrophages upon co-culture with SW1463 cells, of pro-inflammatory *CD80*, a co-stimulatory molecule involved in antigen presentation, could be an attempt of macrophages to overcome their apparent lack of tumouricidal activity on SW1463 cells.

Although our main goal was to explore the importance of macrophage-cancer cell communication in response to radiation, it became relevant also to investigate that crosstalk without radiation exposure. In that respect, several studies start to point some common conclusions, such as both macrophages and cancer cells are mutually affected when in co-culture, and also that macrophages may develop a mixed M1/M2 phenotype, whose exact profile depends on the selected cancer cell line[36]. For instance, supernatants of two CRC lines with different pathological status, HT-29 (Dukes' B stage, meaning invasion into the muscle layer of the bowel) and Colo205 (Dukes' D stage, meaning advanced CRC), induced a more pro-inflammatory (M1-like) or anti-inflammatory (M2-like) phenotype in PMA-treated THP-1 cells, respectively[36]. Accordingly, and

using a similar *in vitro* system to the one we used, Hollmén and colleagues co-cultured, for 5 days, human monocytes with two breast cancer lines. Their data suggested the induction of a pro-inflammatory macrophage phenotype upon co-culture with T47D cells, which are less invasive and respond well to anti-hormonal therapy, and of an anti-inflammatory phenotype upon co-culture with MDA-MB-231 cells, which are highly invasive cancer cells with lack of effective treatment[34,55.] Altogether these studies support our results, demonstrating a mutual influence between macrophages and cancer cells, which, although under non-irradiation conditions, may be crucial for cell response to radiation. However, we cannot exclude that these interactions may be different *in vivo*, where macrophage-cancer cell crosstalk is subjected to the influence of the other cells from the tumour microenvironment.

Finally, by studying how irradiated cells affect those that have not been directly exposed to ionizing radiation, a phenomenon termed as radiation-induced bystander effect[56], we demonstrate that, contrary to non-irradiated SW1463, RKO cells become more invasive and migrate more in the presence of irradiated macrophage-cancer cell co-culture-released signals. This suggests that, although the enhancement of SW1463 radioresistance by macrophages constitutes a motif of concern, attention should also be paid to the non-targeted effects of radiotherapy, particularly those mediated by radiosensitive cells, like RKO.

Conclusions

Altogether this data reinforce our previous results[23], demonstrating that ionizing radiation modulates macrophage profile towards a more pro-inflammatory one and that this is also the case when macrophages are co-cultured with colorectal cancer cells. Furthermore, the molecular crosstalk established between macrophages and cancer cells seems to modulate the response of the latest to ionizing radiation exposure. Remarkably, the present *in vitro* approach demonstrated that macrophages enhance the cancer cell intrinsic response to radiation, promoting RKO radiation sensitivity, while enhancing SW1463 cells radioresistance. This will depend on the intrinsic nature of each cancer cell, how it responds to macrophage presence, as well as on their modulation of macrophage polarization profile (Figure 7). Overall, a better understanding of the mechanisms responsible for cancer cell radioresistance will contribute to the discovery of potential cellular and molecular targets to improve radiotherapy efficacy[57].

Macrophage-cancer cell communication upon irradiation

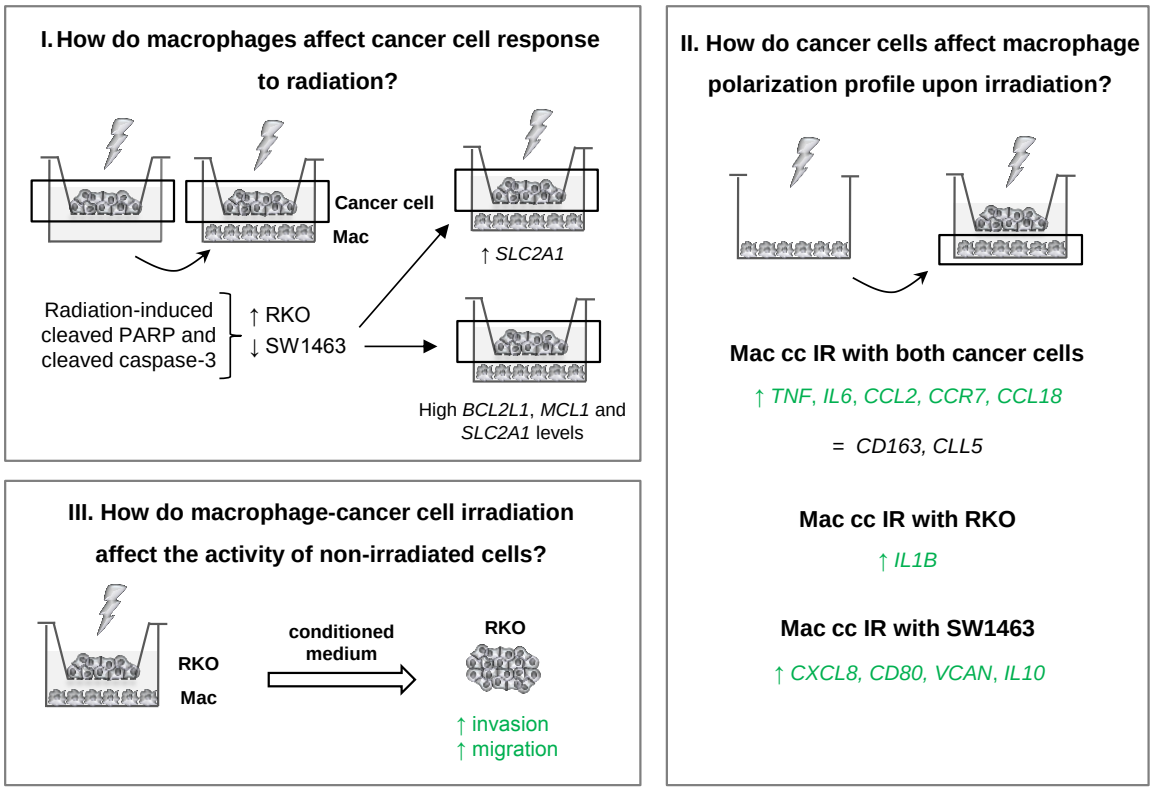

Figure 7 - Intricate macrophage-cancer cell communication upon irradiation. **I)** In the presence of macrophages, radiosensitive RKO cell increased cleaved PARP and cleaved caspase-3 expression in response to radiation, while radioresistant SW1463 reduced the expression of these targets. The high *BCL2L1*, *MCL1* and *SLC2A1* levels observed in SW1463 cells upon co-culture with macrophages, together with the increased expression of *BCL2L1* in irradiated SW1463 upon co-culture with macrophages, may contribute to macrophage-induced SW1463 enhanced radioresistance. **II)** In the presence of either RKO or SW14363 cancer cells, irradiated macrophages exhibit higher levels of pro-inflammatory *TNF*, *IL6*, *CCL2* and *CCR7*, but also of anti-inflammatory *CCL18*, being differences in other targets dependent on the nature of the cancer cells with which macrophages were cultured. Thus, in the presence of RKO cells irradiated macrophages exhibit an increase of pro-inflammatory *IL1B*, while SW1463 cells promote higher pro-inflammatory *CXCL8* and *CD80*, but also anti-inflammatory *VCAN* and *IL10* levels. **III)** Conditioned medium (CM) from macrophage-RKO irradiated co-culture induced increased invasion and migration of non-irradiated RKO cells. Abbreviations: cc – co-cultures; IR – irradiated.

Material and methods

Ethics statement

In the present study, human monocytes were isolated from buffy coats of healthy blood donors, obtained through a collaboration protocol with Centro Hospitalar São João (CHSJ). This was approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. A written informed consent was obtained from all subjects before blood donation. Buffy coats were provided anonymised, and their identification was only accessible to hospital staff.

Human monocyte isolation and macrophage differentiation

Human monocytes were isolated from healthy blood donors as previously described, using the RosetteSep monocyte-enrichment kit (StemCell)[23]. Following this negative separation procedure, over 85% of isolated monocytes were found to be CD14-positive[26]. For monocyte-macrophage differentiation, $1.2x10^6$ cells/9.6 cm^2 were cultured in RPMI1640 medium (with GlutaMax) (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), in the presence of 50 ng/mL of macrophage colony-stimulating factor (M-CSF) (ImmunoTools, Friesoythe, Germany). After 7 days, cell culture medium was replaced without M-CSF renewal.

Cancer cells

Human RKO colon cancer cells were purchased from ATCC, while human SW1463 rectal cancer cells were kindly provided by Prof Kevin M. Haigis (Molecular Pathology Unit, Center for Cancer Research and Center for Systems Biology, Massachusetts General Hospital, USA). Cell DNA was analysed with POWERPLEX 16 HS kit (Promega, Madison, WI, USA) and cell lines were tested and authenticated by autosomal STR DNA profiling at IPATIMUP Diagnostics, a laboratory accredited by the College of American Pathologists and with a Quality Management System certified in accordance with NP EN ISO 9001:2008 (IPATIMUP Diagnostics, Porto, Portugal). Both cell lines were cultured in RPMI1640 medium (with GlutaMax) (Invitrogen) supplemented with 10% FBS (Lonza), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), at 37°C and 5% CO_2.

Establishment of macrophage-cancer cell co-cultures

Eleven days after monocyte isolation, RKO ($12.6x10^3$ cells/well) or SW1463 ($12.8\text{-}16.0x10^4$ cells/well) cancer cells were plated in 6 well-plate permeable transwell inserts (Corning, Cat. No. 353102, New York, USA), and placed on top of macrophages (Fig 1). The permeable PET membrane of 1.0 µm pore size avoided cancer cells to cross from the top to the lower compartment, where

macrophages were previously differentiated, allowing however the exchange of soluble factors between both populations. Co-cultures were maintained in RPMI1640 medium (with GlutaMax) (Invitrogen) supplemented with 10% FBS (Lonza), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) for 3 days before irradiation. Due to limited availability of human primary macrophage material, RKO and SW1463 cells were co-cultured with a different set of macrophages, derived from 4 distinct blood donors, in two independent experiments per each cell line. For control purposes, macrophage and cancer cell monocultures were also prepared.

Ionizing radiation exposure

The dosimetry plan was established, as previously reported[23]. Cell culture medium was renewed before the first irradiation and mono- or co-cultures were exposed to cumulative ionizing radiation doses (2 Gy/fraction/day), for 5 days (5 x 2 Gy), totalizing 10 Gy. Photon beam was produced by a PRIMUS (Siemens, Malvern, PA, USA) linear particle accelerator, used for human radiotherapy, operated at 18 MV at the Radiotherapy Service of CHSJ. To avoid differences between non-irradiated and irradiated cells, caused by medium agitation during transport to/from the Radiotherapy Service, control cells were also transported, but were not exposed to radiation.

Western Blot analysis

Total protein was extracted 6 h after cumulative ionizing radiation doses (5 x 2 Gy). Lysis buffer A [20 mM Tris-HCl (pH 7.5-8), 600 mM NaCl, 1% Igepal], or RIPA [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% Igepal] were supplemented with a cocktail of proteases and phosphatases inhibitors: phenylmethanesulfonylfluoride 1 mM, sodium metavanadate 3 mM, sodium fluoride 20 mM, sodium pyrophosphate tetrabasic 25 mM (Applichem), aprotinin 10 mg/ml and leupeptin 10 mg/ml (Sigma-Aldrich). Proteins extracted with Laemmli buffer 1x [3% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.1% blue bromophenol in 1M Tris-HCl pH 6.8] were sonicated to shear DNA. Protein concentration was determined with Protein Assay Dye Reagent Concentrate (BioRad). Before loading into 10-15% SDS-polyacrylamide gels, proteins were diluted in Laemmli buffer containing β-mercaptoethanol (BioRad) and denatured at 95°C for 5 min. Primary antibodies against cleaved PARP (dilution 1:1000, clone D64E10, Cell Signalling), cleaved caspase-3, caspase-3, phospho-Chk2 (Thr 387) and Chk2 (dilution 1:1000, Cell Signalling) were used. Antibody against β-actin (dilution 1:10000, clone 8H10D10, Cell Signalling) was used to normalize protein expression. Goat anti-rabbit or horse anti-mouse-Horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:2000, Cell Signalling) were used, followed by ECL- detection (Thermo Fisher Scientific).

RNA extraction, cDNA preparation and quantitative PCR analysis

Total RNA was extracted 6 h after irradiation (5 x 2 Gy), using TriPure Isolation Reagent (Roche), according to manufacturer´s instructions. RNA was converted to cDNA as previously described [23]. To evaluate mRNA expression levels, quantitative PCR using Brilliant II SYBR green kit (Stratagene/Agilent Technologies) and MX3005P qPCR platform (Stratagene/Agilent) was performed. The following primers were used for RT-qPCR: CXCL8, F:5'-CCAGGAAGAAACCACCGGA-3', R:5'-GAAATCAGGAAGGCTGCCAAG-3'; IL1B, F:5'-GGCAGGGAACCAGCATC-3', R:5'-CCGACCACCACTACAGCAA-3'; MCL1, F:5´-CAAGCAGAAGTGGGTTCAGGAT-3', 5'-TCTTCGGAGTTTGGGTTTGC-3'; LDHA, F:5'-GGAGATCCATCATCTCTCCC-3´, R:5'-GGCCTGTGCCATCAGTATCT-3' (Invitrogen); BCL2L1, F:5'-CTGCTGCATTGTTCCCATAG-3´, R:5'-TTCAGTGACCTGACATCCCA-3´; SLC2A1, F:5´-CGGGCCAAGAGTGTGCTAAA-3', R:3'-TGACGATACCGGAGCCAATG-5'(Genomic Oligo); CCL5 F:5'-GTCGTCTTTGTCACCCGAAAG-3', R:5'-TCCCGAACCCATTTCTTCTCT-3'. Primer sets for *ACTB* (used as a housekeeping gene) and *CCL2* were obtained from Qiagen, while probes for CD80, CCR7, TNF, IL6, CD163, IL10, CCL18, CSF1 and VCAN were from Applied Biosystems.

Functional assays

To study the effect of conditioned medium (CM) from irradiated co-culture on the functional activity of non-irradiated cells, RKO or SW1463 were stimulated with CM from co-cultures collected 6 h after irradiation (5 x 2 Gy). For proper comparison, RKO cells were stimulated with CM from irradiated or non-irradiated macrophage-RKO co-cultures, while SW1463 were exposed to CM from irradiated or non-irradiated macrophage-SW1463 co-cultures and cancer cell migration and invasion were then evaluated. The angiogenic potential of CM from irradiated or non-irradiated co-cultures was directly evaluated using an *in vivo* model, without cancer cell inoculation.

Matrigel invasion assays

Non-irradiated RKO or SW1463 cells ($5x10^4$) were seeded on the upper compartment of Matrigel-coated inserts with 8-µm pore size (BD Biosciences, Madrid, Spain) and stimulated with CM from irradiated or non-irradiated co-cultures for 24 h, at 37°C and 5% CO_2. The porous membranes were then washed, fixed in 4% paraformaldehyde, mounted in Vectashield+4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) for nuclei staining, and the number of invasive cells was counted on a fluorescence light microscope (Leica).

Migration assay

Non-irradiated RKO or SW1463 cancer cells were plated in individual culture-inserts (Ibidi, Cat. No. 80209, Munich, Germany), appropriated for 2D migration assays, and maintained at 37°C and 5% CO_2 until confluence. These culture inserts were composed by two chambers separated by a biocompatible silicone material, which after removal allowed cells from each edge to migrate towards the centre of the gap. So, after barrier removal, confluent cancer cell monolayers were washed with PBS, to remove non-adherent cells, and stimulated with CM from irradiated or non-irradiated co-cultures. Stimulated cells were maintained at 37°C and 5% CO_2 for 48 h. Cell migration was daily followed with photos acquired with the digital Camera EOS 1000D (Cannon) connected to a brightfield microscope (Zeiss).

Chick embryo chorioallantoic membrane (CAM) angiogenesis assay

Before performing the assay, CM from irradiated and non-irradiated co-cultures were concentrated around 10 times in a Savant SpeedVac Concentrator under vacuum (Thermo Scientific, Massachusetts, EUA). The chick embryo CAM model was used to evaluate the angiogenic potential of both CM, as previously described[23]. On embryonic development day (EDD)10, concentrated CM from irradiated or non-irradiated co-cultures were inoculated on top of the same CAM into two independent 3 mm silicone rings, under sterile conditions. Eggs were re-sealed and returned to the incubator for additional 72 h. On EDD13, rings were removed, the CAM was excised from embryos and photographed *ex-ovo* under a stereoscope, using a 20x magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (< 20 µm diameter) growing radially towards the inoculation area was counted in a blind fashion manner. Eggs from two different batches were inoculated with CM from macrophage-RKO and macrophage-SW1463 co-cultures, obtained from 4 independent experiments (n = 15/condition).

Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v5 (GraphPad-trial version). As the recommended normality test - D'Agostino and Pearson required $n \geq 8$ and the present study only involved comparisons with a maximum of $n = 4$, it was not possible to analyse data for Gaussian distribution. Therefore, *t*-test (paired, non-paired or one sample *t*-test) was used to compare data. Statistical significance was achieved when $P < 0.05$. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

References

1. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer cell. 2012;21(3):309-22. doi:10.1016/j.ccr.2012.02.022.
2. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nature reviews Cancer. 2009;9(4):239-52. doi:10.1038/nrc2618.
3. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124(2):263-6. doi:10.1016/j.cell.2006.01.007.
4. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature reviews Cancer. 2004;4(1):71-8. doi:10.1038/nrc1256.
5. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. Cancer cell. 2013;23(3):277-86. doi:10.1016/j.ccr.2013.02.013.
6. Mantovani A, Allavena P. The interaction of anticancer therapies with tumor-associated macrophages. The Journal of experimental medicine. 2015;212(4):435-45. doi:10.1084/jem.20150295.
7. Delaney G, Jacob S, Featherstone C, Barton M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer. 2005;104(6):1129-37. doi:10.1002/cncr.21324.
8. Hellevik T, Martinez-Zubiaurre I. Radiotherapy and the tumor stroma: the importance of dose and fractionation. Frontiers in oncology. 2014;4:1. doi:10.3389/fonc.2014.00001.
9. Meng Y, Beckett MA, Liang H, Mauceri HJ, van Rooijen N, Cohen KS et al. Blockade of tumor necrosis factor alpha signaling in tumor-associated macrophages as a radiosensitizing strategy. Cancer research. 2010;70(4):1534-43. doi:10.1158/0008-5472.CAN-09-2995.
10. De Ridder M, Verovski VN, Darville MI, Van Den Berge DL, Monsaert C, Eizirik DL et al. Macrophages enhance the radiosensitizing activity of lipid A: a novel role for immune cells in tumor cell radioresponse. International journal of radiation oncology, biology, physics. 2004;60(2):598-606. doi:10.1016/j.ijrobp.2004.05.065.
11. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends in immunology. 2004;25(12):677-86. doi:10.1016/j.it.2004.09.015.
12. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in immunology. 2002;23(11):549-55.
13. Ostuni R, Kratochvill F, Murray PJ, Natoli G. Macrophages and cancer: from mechanisms to therapeutic implications. Trends in immunology. 2015;36(4):229-39. doi:10.1016/j.it.2015.02.004.
14. Lee JM, Bernstein A. p53 mutations increase resistance to ionizing radiation. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(12):5742-6.
15. Servomaa K, Kiuru A, Grenman R, Pekkola-Heino K, Pulkkinen JO, Rytomaa T. p53 mutations associated with increased sensitivity to ionizing radiation in human head and neck cancer cell lines. Cell proliferation. 1996;29(5):219-30.
16. Dokic I, Mairani A, Brons S, Schoell B, Jauch A, Krunic D et al. High resistance to X-rays and therapeutic carbon ions in glioblastoma cells bearing dysfunctional ATM associates with intrinsic chromosomal instability. International journal of radiation biology. 2015;91(2):157-65. doi:10.3109/09553002.2014.937511.
17. Lynam-Lennon N, Reynolds JV, Pidgeon GP, Lysaght J, Marignol L, Maher SG. Alterations in DNA repair efficiency are involved in the radioresistance of esophageal adenocarcinoma. Radiation research. 2010;174(6):703-11. doi:10.1667/RR2295.1.
18. Lee JU, Hosotani R, Wada M, Doi R, Kosiba T, Fujimoto K et al. Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. European journal of cancer. 1999;35(9):1374-80.
19. Lynam-Lennon N, Maher SG, Maguire A, Phelan J, Muldoon C, Reynolds JV et al. Altered mitochondrial function and energy metabolism is associated with a radioresistant phenotype in oesophageal adenocarcinoma. PloS one. 2014;9(6):e100738. doi:10.1371/journal.pone.0100738.
20. Baskar R, Dai J, Wenlong N, Yeo R, Yeoh KW. Biological response of cancer cells to radiation treatment. Frontiers in molecular biosciences. 2014;1:24. doi:10.3389/fmolb.2014.00024.
21. Barcellos-Hoff MH, Park C, Wright EG. Radiation and the microenvironment - tumorigenesis and therapy. Nature reviews Cancer. 2005;5(11):867-75. doi:10.1038/nrc1735.
22. Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nature reviews Cancer. 2015;15(7):409-25. doi:10.1038/nrc3958.
23. Pinto AT, Pinto ML, Cardoso AP, Monteiro C, Pinto MT, Maia AF et al. Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. Scientific reports. 2016;6:18765. doi:10.1038/srep18765.
24. Williams JR, Zhang Y, Zhou H, Gridley DS, Koch CJ, Slater JM et al. Overview of radiosensitivity of human tumor cells to low-dose-rate irradiation. International journal of radiation oncology, biology, physics. 2008;72(3):909-17. doi:10.1016/j.ijrobp.2008.06.1928.

25. Kleiman LB, Krebs AM, Kim SY, Hong TS, Haigis KM. Comparative analysis of radiosensitizers for K-RAS mutant rectal cancers. PloS one. 2013;8(12):e82982. doi:10.1371/journal.pone.0082982.

26. Cardoso AP, Pinto ML, Pinto AT, Oliveira MI, Pinto MT, Goncalves R et al. Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. Oncogene. 2014;33(16):2123-33. doi:10.1038/onc.2013.154.

27. Zannini L, Delia D, Buscemi G. CHK2 kinase in the DNA damage response and beyond. Journal of molecular cell biology. 2014;6(6):442-57. doi:10.1093/jmcb/mju045.

28. Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer letters. 2012;327(1-2):48-60. doi:10.1016/j.canlet.2011.12.012.

29. Li HH, Wang YW, Chen R, Zhou B, Ashwell JD, Fornace AJ, Jr. Ionizing Radiation Impairs T Cell Activation by Affecting Metabolic Reprogramming. International journal of biological sciences. 2015;11(7):726-36. doi:10.7150/ijbs.12009.

30. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nature reviews Cancer. 2004;4(11):891-9. doi:10.1038/nrc1478.

31. Shen Z, Seppanen H, Vainionpaa S, Ye Y, Wang S, Mustonen H et al. IL10, IL11, IL18 are differently expressed in CD14+ TAMs and play different role in regulating the invasion of gastric cancer cells under hypoxia. Cytokine. 2012;59(2):352-7. doi:10.1016/j.cyto.2012.04.033.

32. Zhang Y, Sime W, Juhas M, Sjolander A. Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. European journal of cancer. 2013;49(15):3320-34. doi:10.1016/j.ejca.2013.06.005.

33. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC cancer. 2015;15:577. doi:10.1186/s12885-015-1546-9.

34. Hollmen M, Roudnicky F, Karaman S, Detmar M. Characterization of macrophage--cancer cell crosstalk in estrogen receptor positive and triple-negative breast cancer. Scientific reports. 2015;5:9188. doi:10.1038/srep09188.

35. Wang H, Shao Q, Sun J, Ma C, Gao W, Wang Q et al. Interactions between colon cancer cells and tumor-infiltrated macrophages depending on cancer cell-derived colony stimulating factor 1. OncoImmunology. 2016.

36. Wu TH, Li YY, Wu TL, Chang JW, Chou WC, Hsieh LL et al. Culture supernatants of different colon cancer cell lines induce specific phenotype switching and functional alteration of THP-1 cells. Cellular immunology. 2014;290(1):107-15. doi:10.1016/j.cellimm.2014.05.015.

37. Zhou N, Zhang Y, Zhang X, Lei Z, Hu R, Li H et al. Exposure of tumor-associated macrophages to apoptotic MCF-7 cells promotes breast cancer growth and metastasis. International journal of molecular sciences. 2015;16(6):11966-82. doi:10.3390/ijms160611966.

38. Kohro T, Tanaka T, Murakami T, Wada Y, Aburatani H, Hamakubo T et al. A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. Journal of atherosclerosis and thrombosis. 2004;11(2):88-97.

39. Mouradov D, Sloggett C, Jorissen RN, Love CG, Li S, Burgess AW et al. Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. Cancer research. 2014;74(12):3238-47. doi:10.1158/0008-5472.CAN-14-0013.

40. Gatti RA, Berkel I, Boder E, Braedt G, Charmley P, Concannon P et al. Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. Nature. 1988;336(6199):577-80. doi:10.1038/336577a0.

41. Aldridge DR, Radford IR. Explaining differences in sensitivity to killing by ionizing radiation between human lymphoid cell lines. Cancer research. 1998;58(13):2817-24.

42. Minjgee M, Toulany M, Kehlbach R, Giehl K, Rodemann HP. K-RAS(V12) induces autocrine production of EGFR ligands and mediates radioresistance through EGFR-dependent Akt signaling and activation of DNA-PKcs. International journal of radiation oncology, biology, physics. 2011;81(5):1506-14. doi:10.1016/j.ijrobp.2011.05.057.

43. Gupta AK, Bakanauskas VJ, McKenna WG, Bernhard EJ, Muschel RJ. Ras regulation of radioresistance in cell culture. Methods in enzymology. 2001;333:284-90.

44. Shimura T, Noma N, Sano Y, Ochiai Y, Oikawa T, Fukumoto M et al. AKT-mediated enhanced aerobic glycolysis causes acquired radioresistance by human tumor cells. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2014;112(2):302-7. doi:10.1016/j.radonc.2014.07.015.

45. Fujibayashi Y, Waki A, Sakahara H, Konishi J, Yonekura Y, Ishii Y et al. Transient increase in glycolytic metabolism in cultured tumor cells immediately after exposure to ionizing radiation: from gene expression to deoxyglucose uptake. Radiation research. 1997;147(6):729-34.

46. Zhan Q, Bieszczad CK, Bae I, Fornace AJ, Jr., Craig RW. Induction of BCL2 family member MCL1 as an early response to DNA damage. Oncogene. 1997;14(9):1031-9. doi:10.1038/sj.onc.1200927.

47. Reynolds JE, Yang T, Qian L, Jenkinson JD, Zhou P, Eastman A et al. Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer research. 1994;54(24):6348-52.

48. Stephenson-Famy A, Marks J, Suresh A, Caritis SN, Simhan H, Jeyasuria P et al. Antiapoptotic signaling via MCL1 confers resistance to caspase-3-mediated apoptotic cell death in the pregnant human uterine myocyte. Molecular endocrinology. 2012;26(2):320-30. doi:10.1210/me.2011-1282.
49. Song L, Coppola D, Livingston S, Cress D, Haura EB. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer biology & therapy. 2005;4(3):267-76.
50. Morel C, Carlson SM, White FM, Davis RJ. Mcl-1 integrates the opposing actions of signaling pathways that mediate survival and apoptosis. Molecular and cellular biology. 2009;29(14):3845-52. doi:10.1128/MCB.00279-09.
51. Yang J, Sun M, Zhang A, Lv C, De W, Wang Z. Adenovirus-mediated siRNA targeting Bcl-xL inhibits proliferation, reduces invasion and enhances radiosensitivity of human colorectal cancer cells. World journal of surgical oncology. 2011;9:117. doi:10.1186/1477-7819-9-117.
52. Haabeth OA, Lorvik KB, Yagita H, Bogen B, Corthay A. Interleukin-1 is required for cancer eradication mediated by tumor-specific Th1 cells. Oncoimmunology. 2016;5(1):e1039763. doi:10.1080/2162402X.2015.1039763.
53. Wang R, Lu M, Zhang J, Chen S, Luo X, Qin Y et al. Increased IL-10 mRNA expression in tumor-associated macrophage correlated with late stage of lung cancer. Journal of experimental & clinical cancer research : CR. 2011;30:62. doi:10.1186/1756-9966-30-62.
54. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer cell. 2014;26(5):623-37. doi:10.1016/j.ccell.2014.09.006.
55. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell. 2006;10(6):515-27. doi:10.1016/j.ccr.2006.10.008.
56. Mothersill C, Seymour C. Radiation-induced bystander effects, carcinogenesis and models. Oncogene. 2003;22(45):7028-33. doi:10.1038/sj.onc.1206882.
57. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. Nature reviews Cancer. 2011;11(4):239-53. doi:10.1038/nrc3007.

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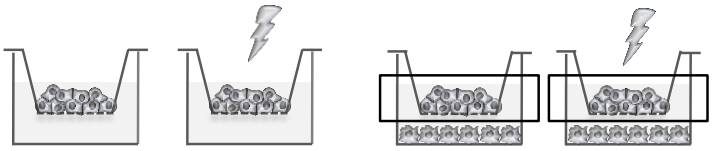
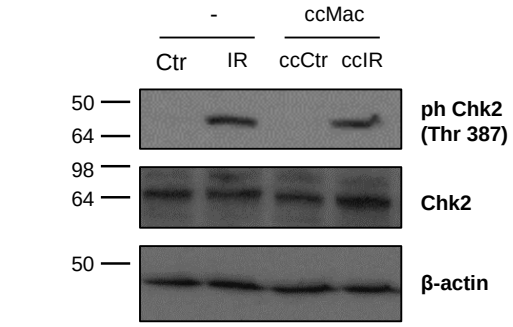
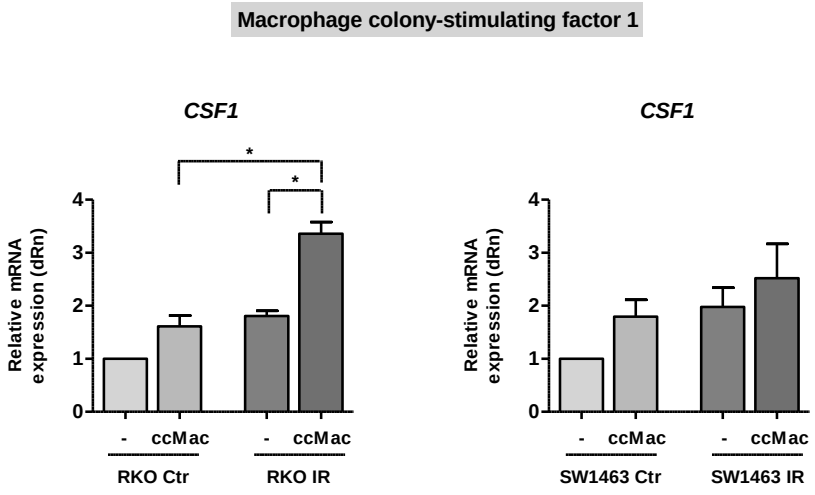
Supporting information





Supplementary Figure S1 - Evaluation of Chk2 phosphorylation levels in SW1463 cancer cells. SW1463 cells were irradiated (IR, 5 x 2 Gy) alone (-) or in co-culture with macrophages (ccMac). Chk2 phosphorylation (Thr 387) and total Chk2 were evaluated, by western blot analysis, 6 h after irradiation.



Supplementary Figure S2 - Evaluation of CSF1 mRNA expression in RKO and SW1463 cancer cells. Both RKO and SW1463 cancer cells were cultured alone (-) or in the presence of macrophages (ccMac), with (IR, 5 x 2 Gy) or without (Ctr) radiation exposure. CSF1 mRNA expression levels were evaluated in cancer cells, 6 h after irradiation. Graphs result from the relative mRNA quantification in cancer cells cultured with macrophages from distinct donors (n = 4 per each cell line), evaluated in four independent experiments. * $P < 0.05$.

Complementary unpublished results

Exploring macrophage-cancer cell co-culture upon irradiation

The following experiment complement those concerning the effect of radiation on macrophage-cancer cell crosstalk, as presented in the manuscript of the present chapter. They involve the same methodology, this is, the indirect co-culture, using transwell inserts, of macrophages with RKO or SW1463 colorectal cancer cell lines, followed by radiation exposure (5 x 2 Gy).

Glucose uptake and lactate levels

As demonstrated in the research article 3, metabolic alterations, particularly at glycolytic pathway, may occur in response to radiation. As a complement, we measured glucose uptake levels in CM from irradiated or non-irradiated macrophage-cancer cell co-cultures (Figure C1).

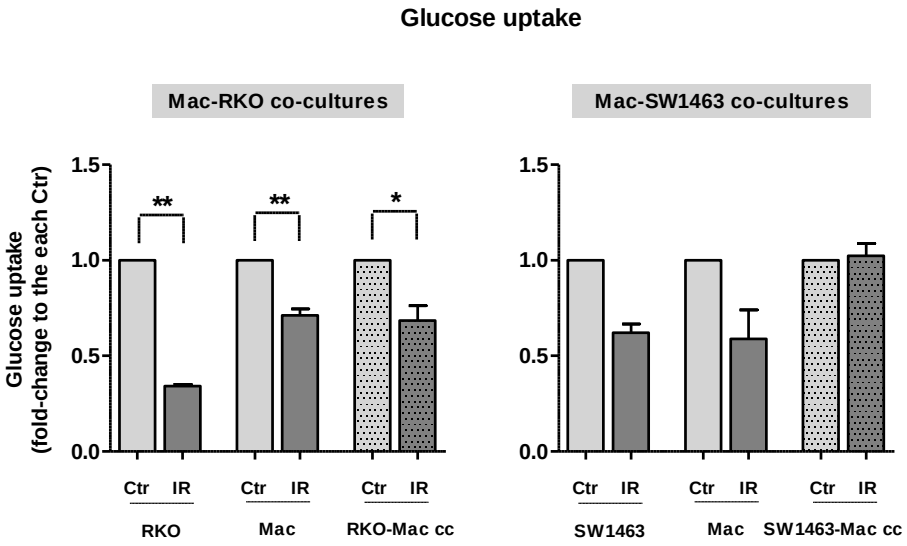


Figure C1 – Glucose uptake decreases in irradiated RKO-macrophage, but not in SW1463-macrophage, co-culture. Indirect co-cultures (cc) of macrophages with RKO or SW1463 colorectal cancer cells, were irradiated (IR) with 10 Gy cumulative ionizing radiation dose and compared with non-irradiated counterparts (Ctr). Glucose uptake was obtained after subtraction of glucose levels measured 6 h after irradiation to the ones originally quantified in RPMI 1640 medium.

Results demonstrate that co-culture of macrophages with RKO, but not with SW1463, exhibited reduced glucose uptake upon exposure to 10 Gy. Additionally, RKO, SW1463 and macrophage monocultures also exhibited reduced glucose uptake upon irradiation, although a higher reduction was observed for RKO.

Exploring irradiated macrophages-mediated bystander effect

Although the results described in the manuscript presented in this chapter were mainly obtained through the establishment and posterior irradiation of macrophage-cancer cell co-cultures, our study of macrophage-cancer cell communication started with the evaluation of the effect of irradiated macrophages on non-irradiated cells, as an example of radiation-induced bystander effect. DNA damage, apoptosis and NK-κB signalling were evaluated in non-irradiated SW480 cells upon stimulation with conditioned medium (CM) from irradiated or non-irradiated macrophages.

DNA damage

DNA damage induction is the classical effect of irradiated cells on non-irradiated ones[1]. Thus, we focused on the evaluation of the phosphorylation status of some molecules associated with DNA damage response, particularly p53, Chk1 and Chk2, in non-irradiated cells after stimulation with CM from irradiated macrophages. As a positive control of the activation of the aforementioned targets, SW480 cells were also stimulated with doxorubicin (2 μM), which is a cytostatic chemotherapeutic agent.

As SW480 cells were cultured in DMEM and macrophages were previously cultured in RPMI, we first checked if there was any effect of adding RPMI medium to SW480 cells (Figure C2A). Our results confirmed that RPMI medium was not exerting, by itself, any effect on the phosphorylation status of Chk1, Chk2 or p53. Additionally, we confirmed that the cytostatic agent doxorubicin was inducing p53 and Chk2 phosphorylation, independently of the duration of the stimulus and that doxorubicin-induced Chk1 phosphorylation was only achieved upon 6 and 24 h of stimulation.

In contrast, we concluded that CM of neither irradiated nor non-irradiated macrophages was inducing Chk1 phosphorylation in SW480 cells (Figure C2B). Also, the Chk2 and p53 phosphorylation levels were quite similar between cells unstimulated or stimulated with both types of CM. These results were consistent, independently of the ionizing radiation dose (2, 6 (3 x 2) or 10 (5 x 2) Gy cumulative dose) received or the duration of the stimulation (1, 4, 6 and 24 h).

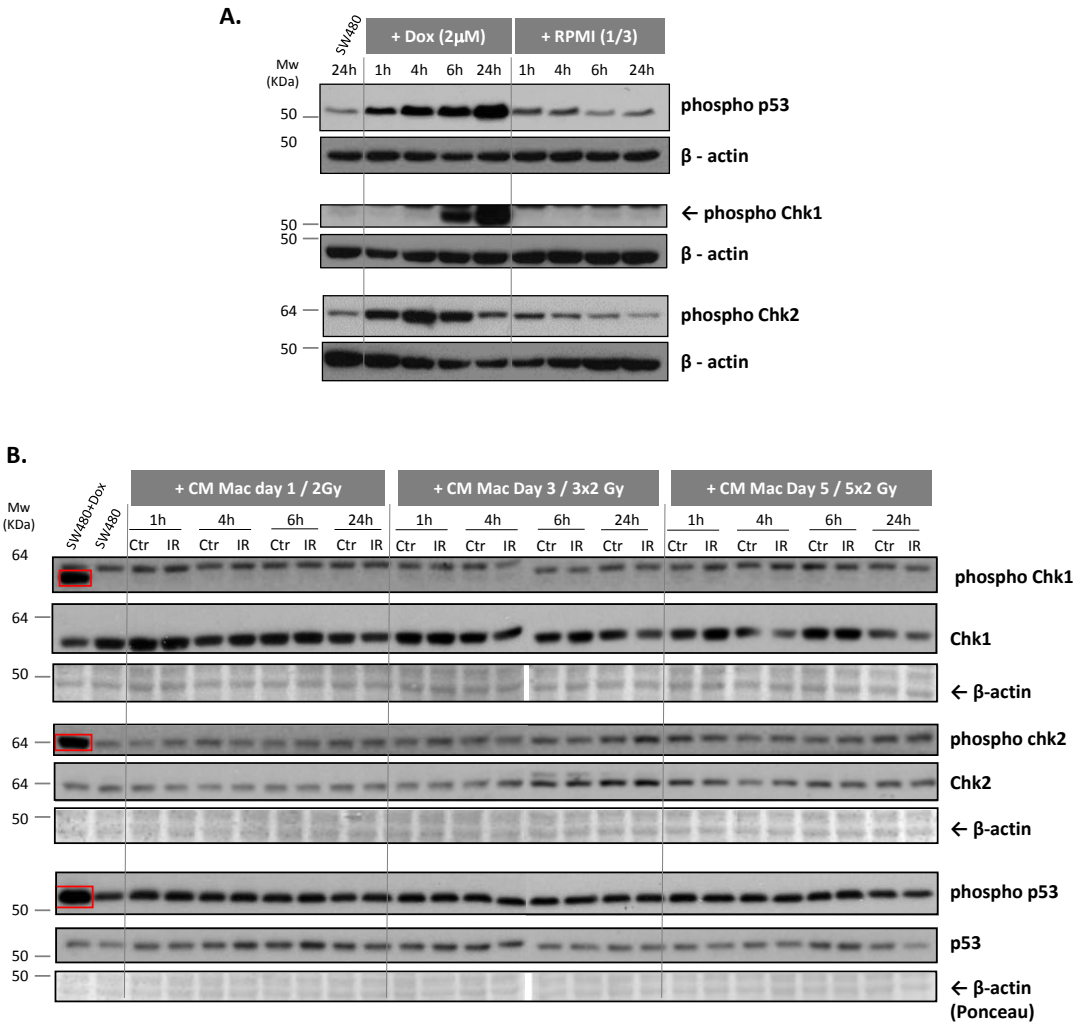


Figure C2 - Conditioned medium from irradiated macrophages do not induce Chk1, Chk2 nor p53 phosphorylation in SW480 colorectal cancer cells. SW480 cells were stimulated for 1, 4, 6 and 24 h with doxorubicin (2 µM), RPMI, or CM from irradiated (2, 6 (3 x 2) and 10 (5 x 2) Gy cumulative doses) or non-irradiated macrophages (*n* = 2), corresponding RPMI and CM to one third of total volume. **A)** Phosphorylation status of Chk1, Chk2 and p53 in SW480 cells stimulated either with doxorubicin (positive control) or RPMI. **B)** Phosphorylation status of Chk1, Chk2 and p53 in SW480 cells stimulated with CM from irradiated or non-irradiated macrophages.

Apoptosis

We then asked whether CM from irradiated macrophages was able to induce apoptosis of SW480 cells. Thus, we evaluated caspases-3 and -7 expression as well as PARP cleavage in SW480 cells exposed to CM from irradiated or non-irradiated macrophages (Figure C3). Although the evaluation of cleaved form of both caspases would have been a clearer indicator of apoptosis induction, an eventual reduction of their total expression levels would also suggest the increase of the cleaved and thereby activated caspase form.

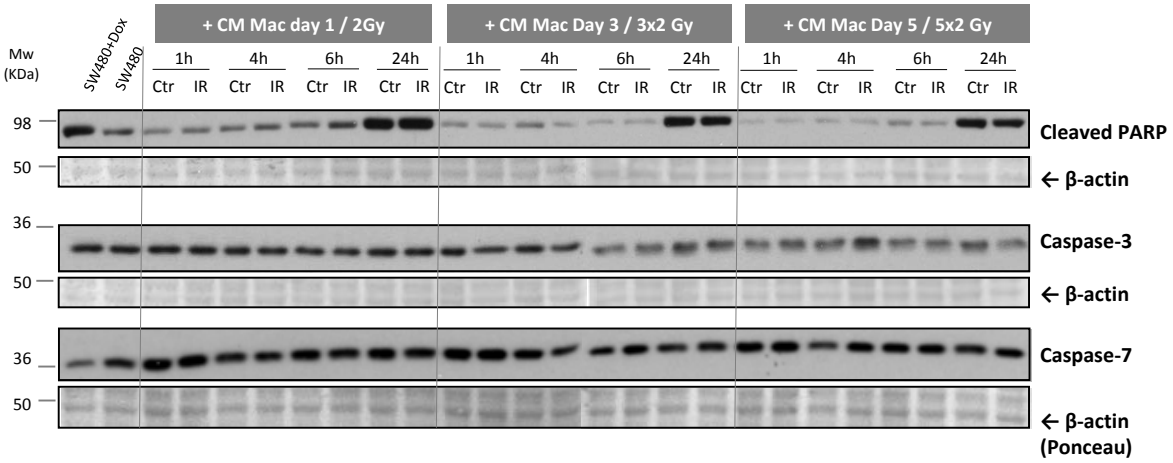


Figure C3 - Stimulation of SW480 cells with conditioned medium from irradiated macrophages does not induce additional alterations in apoptosis-related molecules. SW480 cells were stimulated for 1, 4, 6 or 24 h with CM from irradiated (2, 6 (3 x 2) or 10 (5 x 2) Gy cumulative doses) or non-irradiated macrophages. PARP cleavage as well as total expression levels of caspase-3 and -7 were then evaluated. SW480 cells stimulated with doxorubicin (2 μM) were used as a positive control.

Our results evidenced that stimulation of SW480 colorectal cancer cells with CM from irradiated macrophages did not alter the expression of caspase-3 and -7 nor even increased PARP cleavage, when compared with stimulation with CM from non-irradiated macrophages. However, CM from both irradiated and non-irradiated macrophages increased SW480 PARP cleavage 24 h after stimulation, similarly to exposure to doxorubicin, suggesting some cytotoxicity level associated with the duration of these stimuli.

NF-κB signalling

Although the previous experiments demonstrated no effect of CM from irradiated macrophages on SW480 DNA damage and apoptosis induction, suggesting no stress response, we complemented this data by evaluating NF-κB signalling pathway activation. Therefore, the phosphorylation levels of IκBα, and of two NF-κB subunits, RelB and p52 (as well as its precursor, the p100), were evaluated in SW480 after exposure to CM from irradiated or non-irradiated macrophages (Figure C4).

These experiments suggested that CM from irradiated macrophages was not inducing significant alterations in NF-κB p100/p52 and RelB subunit expression and on IκBα phosphorylation, reinforcing that this stimulus was not generating a stress response in SW480 cells.

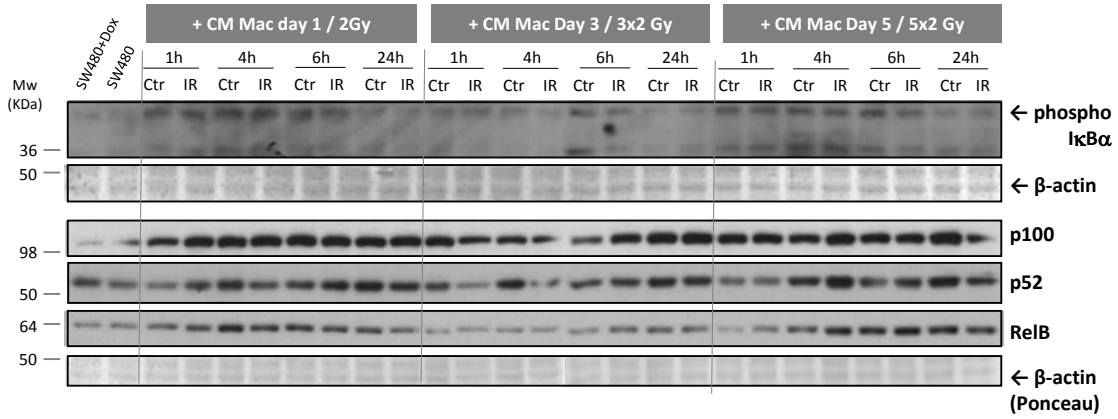


Figure C4 – Both conditioned medium from irradiated and non-irradiated macrophages are not inducing significant alterations in NF-κB p100/p52and RelB subunits expression and IκBα phosphorylation in SW480 cells. SW480 cells were stimulated for 1, 4, 6 or 24 h with CM from irradiated (2, 6 (3 x 2) or 10 (5 x 2) Gy cumulative dose) or non-irradiated macrophages. Phosphorylated IκBα and total expression of NF-κB p100, p52 and RelB subunits were evaluated.

Overall, conditioned medium from irradiated macrophages did not induce major alterations regarding DNA damage induction, apoptosis and NF-kB signalling activation in non-irradiated SW480 colorectal cancer cells. This lack of major radiation-induced bystander effect by irradiated macrophages could be attributed to their radioresistant profile. Probably, macrophages are not releasing danger signals to the supernatant, maintaining the viability of cancer cells exposed to this stimulus. These preliminary results led us to establish indirect macrophage-cancer cell co-cultures to better understand the communication between both populations upon irradiation, as described in the manuscript of the present chapter. Altogether, data from our research article 3 and these complementary results reinforce the importance of using methodological approaches allowing the contact, even in an indirect manner, between macrophages and cancer cells to reveal how this crosstalk influences the response of both populations to external stimuli, such as ionizing radiation.

References

1. Nagasawa, H. & Little, J.B. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer research* **52**, 6394-6396 (1992).

Chapter summary

Our main achievements, regarding the macrophage-cancer cell communication upon exposure to 5 cumulative ionizing radiation doses (**Research Article 3**), are summarized as followed:

- the presence of macrophages reduces radiation-induced apoptosis in SW1463, but not in RKO cells, as demonstrated by evaluation of caspase-3 and PARP cleavage;
- SW1463 cancer cells enhanced radioresistance in the presence of macrophages does not seem to be attributed to a reduction of ionizing radiation-induced DNA damage signalling, but could be rather associated with an increased expression of survival- and metabolism-related genes upon co-culture with macrophages;
- in response to radiation, the presence of macrophages induced upregulation of *CSF1* in RKO, but not in SW1463 cells;
- colorectal cancer cells promote a macrophage pro-inflammatory phenotype, with or without radiation exposure, as indicated by evaluation of the expression of a panel of pro- and anti-inflammatory markers. Particularly, irradiated macrophages exhibit increased expression of the pro-inflammatory *IL1B,* upon co-culture with RKO cells, or increased expression of the pro-inflammatory *CD80* and anti-inflammatory *IL10*, upon co-culture with SW1463 cells;
- conditioned medium from irradiated macrophage-RKO co-culture increased migration and invasion of non-irradiated RKO cells

Additionally (**complementary unpublished results**), we have also demonstrated that:

- RKO-macrophage, but not SW1463-macrophage, co-cultures exhibited reduced glucose uptake;
- conditioned medium from irradiated macrophages does not induce major alterations regarding DNA damage induction, apoptosis and NF-kB signalling activation in non-irradiated SW480 colorectal cancer cells.

Chapter VI

Concluding remarks and future perspectives

Radiotherapy is a widely used anti-cancer therapy, being particularly relevant for the management of certain types of cancer, including (colo)rectal cancer. Since X-ray discovery in 1895, we have assisted to a huge evolution in the field, from exploration of radiation physics, to radiobiology studies, technological advances in imaging and treatment planning, and ultimately to the discovery of new molecular targets for radiosensitization[1]. Despite the contribution of several sciences and tools for a better understanding of cellular effects of radiation exposure and increased precision and accuracy of radiation treatment, there is still room for improvement, particularly in what concerns the control of local recurrences and distant disease.

Although some biological strategies still focus solely on cancer cells, others aim to target the tumour microenvironment, by modifying the interactions between cancer cells and the network of stromal cells and ECM that supports tumour growth and resistance to therapy. Particularly macrophages, one of the most abundant immune population at the tumour microenvironment, may contribute to cancer cell invasion, migration, angiogenesis and metastasis[2], also promoting cancer resistance to radiotherapy[3]. However, more research is still required to better characterize macrophage response to radiation, particularly at functional and molecular levels, as well as to better understand the mechanisms responsible for macrophage-mediated cancer cell resistance to radiotherapy. This knowledge is crucial for the development of new biological strategies that could be delivered concomitantly, before or after radiation treatment in order to improve radiotherapy efficacy.

So far, the main limitations to address these issues are the persistent use of murine macrophages, either cell lines or bone-marrow derived ones, and the frequent use of low or high single doses, which together do not allow the parallelism with human macrophage biology nor with the clinical situation of fractionated radiotherapy. Although the delivery of high-doses, using either single or fractionated doses, may be used in some radiotherapy techniques, such as stereotactic body radiation therapy (SBRT) for the treatment of extracranial tumours, single doses of 1.8-2 Gy still constitute the conventional fractionated radiotherapy scheme[4]. Thus, the novelty of our work relies on the use of human monocyte-derived macrophages, instead of cell lines, and on cell exposure to fractionated ionizing radiation doses, mimicking 1 week of a (colo)rectal cancer patient´s treatment (5 x 2 Gy), rather than to single doses.

Therefore, the present PhD thesis aimed to contribute with new insights into the biological response of macrophages to radiation, either from a functional (Chapter III) or signalling point of views (Chapter IV), and also into the comprehension of macrophage-cancer cell communication upon radiation exposure (Chapter V). Our first data (Chapter III) corroborate previous studies,

reporting a macrophage radioresistant phenotype[5, 6]. We demonstrated that despite DNA damage, macrophages maintained their metabolic activity upon exposure to 10 Gy cumulative ionizing radiation doses, which is probably sustained by increased expression of pro-survival molecules, such as Bcl-xL (AT Pinto, 2016, *published at Scientific Reports*)[7]. Our results demonstrated that ionizing radiation increased macrophage phagocytosis, promoted NF-κB activation and increased expression of pro-inflammatory molecules, such as *CD80*, CD86 and HLA-DR. Additionally, we also observed a reduction of the anti-inflammatory markers IL-10, *CD163* and *MRC1* in irradiated macrophages. However, a complete pro-inflammatory macrophage phenotype was not fully achieved, as some features characteristic of anti-inflammatory macrophages, such as high MMP-9 activity, macrophage-cancer cell mediated invasion and tumour angiogenesis, were still observed. As a result of the discovery of NF-κB overexpression, particularly of the RelB subunit, upon macrophage irradiation, it could be interesting to target it and to evaluate whether that inhibition was able to modulate macrophage resistance to therapy and also counteract macrophage-mediated cancer cell invasion upon irradiation. Due to its role in a variety of cellular responses, namely stress conditions and inflammation, a plethora of agents, like natural products, chemicals, synthetic compounds, peptides, and physical conditions, was found to inhibit NF-κB activity at different levels, from upstream kinases to NF-κB transactivation[8]. However, many of these inhibitors, such as antioxidants, display elevated unspecific activity, targeting other pathways besides NF-κB, or frequently lead to inhibition of the most common NF-κB subunit – the RelA[8]. However, the discovery of RelB overexpression in irradiated prostate cancer cells led to the investigation of specific RelB targeting strategies, namely siRNA inhibitors, vitamin D3 and a novel NF-κB inhibitor, a cell permeable peptide (SN52) able to block the nuclear import of RelB:p52 dimer, which were all reported to inhibit RelB and promote radiosensitization of prostate cells, probably through reduction of RelB-induced antioxidant and anti-apoptotic MnSOD[9, 10, 11]. Although the development of alternative and more recently optimized strategies, including new transfection reagents, it is important to mention that traditional RNAi methods for gene knock-down have low efficiency in macrophages[12, 13].

In addition to the described phenotype for irradiated macrophages, our proteomic study (Chapter IV) also revealed that irradiated macrophages presented downregulation of several biological processes, including *protein folding in endoplasmic reticulum* (AT Pinto, 2016, manuscript conditionally accepted at *Journal of Proteome Research* 2016). Contrarily to macrophages, radiosensitive endothelial cells upregulate GRP78, which is an ER chaperone that helps in the refolding of abnormal proteins, and increase endoplasmic reticulum stress and cellular apoptosis after radiation exposure[14]. Additionally, these radiosensitive cells also exhibit a senescence-like

phenotype upon irradiation, confirmed by several features, namely an increase of β-galactosidase activity[15]. The evaluation of this widely used biomarker of cell senescence[16] would probably reveal that, in opposite to endothelial cells, macrophages do not exhibit cellular senescence upon radiation exposure. Also the evaluation of autophagy activation, as a stress response, could be interesting to explore, but our preliminary results demonstrated no increase of the autophagy marker protein LC3B (data not shown) in irradiated macrophages.

Data from proteomic analysis of irradiated macrophages also evidenced expression alterations of two main targets, cathepsin D, a lysosomal aspartyl protease involved in apoptosis and antigen processing, and transferrin receptor 1 (CD71), a mediator of iron uptake. Focusing on cathepsin D, several experiments can be further performed to explore its role in irradiated macrophages. Particularly, i) the verification of cathepsin D activation, ii) the analysis of MHC-class II expression levels in irradiated macrophages, and iii) the evaluation of the efficiency of antigen processing and presentation. The last one can be indirectly determined by T cell activation, through evaluation of released IL-2 cytokine levels in T cells supernatants, after co-culture of irradiated macrophages with cytotoxic T cells[17, 18]. Of major importance to decipher the role of cathepsin D in irradiated macrophages, would be to overexpress it and evaluate macrophage apoptosis as well as the efficiency of antigen processing and presentation. Cathepsin D activation is known to mediate cytochrome c release and caspase activation in different cell types in response to stress conditions, such as staurosporine or oxidative stress[19, 20], while cathepsin D downregulation prevents chemotherapy-induced apoptosis[21]. Importantly, the evaluation of cathepsin D and transferrin receptor expression in tumours from patients subjected to radiotherapy, should also be considered. Overall, data presented in Chapters III-IV described a very complete functional and signalling pathway profile for macrophages exposed to fractionated ionizing radiation doses, as was never reported before. Additionally, our data reinforces macrophage survival and adaptive phenotype upon irradiation.

As macrophages may contribute to cancer progression and response to therapy, it became pertinent to explore the macrophage-colorectal cancer cell crosstalk upon radiation exposure. To address this issue, we invested on the establishment of indirect co-cultures between macrophages and cancer cells, which allowed mimicking the communication between macrophages and cancer cells at the tumour microenvironment. Irradiation of these co-cultures demonstrated that macrophages were able to promote the radiosensitivity of RKO cells, while enhancing SW1463 radioresistance (AT Pinto, 2016, manuscript *submitted to PLOS ONE*). These observations could be due to a different modulation of macrophage response to radiation, or to the intrinsic properties of each cell line, namely radiosensitivity/radioresistance profile, or to a combination of both.

Intrinsic nature of cancer cells is of considerable importance for response to radiation. For instance, the implantation of a tumour cell line transfected with a double-strand break repair gene (*DNA-PKcs*) reduced mice radiosensitivity and caused a decrease in tumour growth delay upon irradiation[22]. However, the doubling time of irradiated regrowing tumours was higher when cell lines were implanted in SCID mice, which exhibit a particular vascular radiosensitivity, compared with nude mice[23]. This suggests that besides tumour cell radiosensitivity, stromal cell response to radiation also influences treatment outcome. Accordingly, several works have evidenced the ability of stromal cells, like fibroblasts or endothelial cells, to enhance cancer cell radioresistance[24, 25]. To further explore the importance of cancer cell genetic alterations or of macrophage phenotype in cancer cell response to radiation, it would be interesting i) to overexpress or silence certain mutated genes in cancer cells or ii) to exogenously induce a pro-inflammatory macrophage polarization profile, before macrophage-cancer cell co-culture irradiation. Pro-inflammatory mouse macrophages, obtained through LPS and IFN-γ stimulation, are known to radiosensitize cancer cells, involving iNOS-mediated macrophage NO synthesis, possibly associated with reduced oxygen consumption in cancer cells[26]. However, as human macrophages have considerable differences when compared with mouse ones, namely NO production[27], basic research aiming to clarify the role of macrophages in cancer cell response to radiation is still required.

In summary, our study demonstrated that macrophages exposed to cumulative ionizing radiation doses up to 10 Gy are viable, enhancing pro-survival activity. Additionally, irradiated macrophages are biologically active, maintaining their ability to respond to exogenous pro- and anti-inflammatory stimuli. Although radiation seems to partially direct macrophages towards a more pro-inflammatory phenotype, which could enhance cancer cell cytotoxicity and therefore increase radiotherapy efficacy, we demonstrated that irradiated macrophages may still promote cancer cell invasion (Chapter III), which is a matter of concern. This data suggests that macrophage expression or release of pro-invasive factors is probably not being affected by ionizing radiation exposure. However, further experiments involving inhibition of EGF-like molecules, which are known to play a role in cancer cell invasion, could clarify whether the mechanisms responsible for macrophage mediated-cancer cell invasion are similar with and without radiation exposure. Additionally, a better comprehension of the biological processes associated with macrophage radioresistance ultimately allows its modulation, which may have an impact in cancer cell response to radiation. We hypothesized that cathepsin D downregulation may have a role in the absence of apoptosis signalling activation observed in irradiated macrophages (Chapter IV), although further experiments are required to address this issue. Additionally, as we also found

that macrophages seem to enhance the intrinsic cancer cell response to radiation (Chapter V), it would be interesting to explore the role of cathepsin D in the modulation of macrophage-cancer cell crosstalk upon irradiation. Our results also demonstrated that irradiated macrophage-cancer cell co-culture could increase the invasion of non-irradiated cancer cells. This finding is of major concern, particularly when making the parallelism with the *in vivo* situation considering, for instance, the irradiated co-cultures as the primary tumour subjected to radiotherapy, and the non-target cells as metastatic cancer cells, not exposed to radiation. Therefore, this result questions whether radiation-induced bystander effects should somehow be taken into consideration. Finally, further experimental work using *in vivo* models could be essential to reveal whether the described biological effects also occur in irradiated tumours, where the crosstalk between macrophages and cancer cells is much more complex, as it involves several players.

To complement the research work presented in Chapters III-V, we believe it is crucial to further validate our results, such as cathepsin D downregulation, in human tumours. Additionally, the characterization of macrophage infiltration density, pattern distribution and phenotype upon tumour irradiation is of major importance, as it may correlate with patients' clinical outcome, thereby constituting a powerful tool to predict tumour response to radiotherapy. To address these issues, the histological comparison of macrophages, from the same cancer patient, before (tumour biopsy) and after (tumour surgical piece) radiotherapy, is required. Biopsies are obtained at the time of the diagnosis, while tumour pieces may be excised during surgery when this is proceeded by the radiation treatment. This study has indeed been initiated, in close collaboration with medical doctors from Pathology and Radiotherapy Services from HSJ (Porto), through the collection and evaluation of available clinical cases, particularly of rectal cancer patients. However, the immunohistochemistry analysis was not further conducted due to several technical limitations, namely the reduced amount of tissue in tumour biopsies, which would compromise an appropriate comparative study.

Overall, the present PhD work contributes with new insights into the response of both macrophages and cancer cells to fractionated ionizing radiation doses, using a similar radiotherapy scheme to the one employed during cancer patients' treatment (2 Gy/fraction/day). Additionally, our results also evidence the importance of macrophages in cancer cell response to radiation, reinforcing their use as important immune targets to improve radiotherapy efficacy.

References

1. Bernier, J., Hall, E.J. & Giaccia, A. Radiation oncology: a century of achievements. Nat Rev Cancer 4, 737-747 (2004).
2. Pollard, J.W. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4, 71-78 (2004).
3. De Palma, M. & Lewis, C.E. Macrophage regulation of tumor responses to anticancer therapies. Cancer cell 23, 277-286 (2013).
4. Laine, A.M. et al. The Role of Hypofractionated Radiation Therapy with Photons, Protons, and Heavy Ions for Treating Extracranial Lesions. Frontiers in oncology 5, 302 (2015).
5. Perkins, E.H., Nettesheim, P. & Morita, T. Radioresistance of the engulfing and degradative capacities of peritoneal phagocytes to kiloroentgen x-ray doses. Journal of the Reticuloendothelial Society 3, 71-82 (1966).
6. Gallin, E.K., Green, S.W. & Sheehy, P.A. Enhanced activity of the macrophage-like cell line J774.1 following exposure to gamma radiation. Journal of leukocyte biology 38, 369-381 (1985).
7. Pinto, A.T. et al. Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. Scientific reports 6, 18765 (2016).
8. Gilmore, T.D. & Herscovitch, M. Inhibitors of NF-kappaB signaling: 785 and counting. Oncogene 25, 6887-6899 (2006).
9. Josson, S. et al. RelB regulates manganese superoxide dismutase gene and resistance to ionizing radiation of prostate cancer cells. Oncogene 25, 1554-1559 (2006).
10. Xu, Y. et al. Suppression of RelB-mediated manganese superoxide dismutase expression reveals a primary mechanism for radiosensitization effect of 1alpha,25-dihydroxyvitamin D(3) in prostate cancer cells. Molecular cancer therapeutics 6, 2048-2056 (2007).
11. Xu, Y. et al. SN52, a novel nuclear factor-kappaB inhibitor, blocks nuclear import of RelB:p52 dimer and sensitizes prostate cancer cells to ionizing radiation. Molecular cancer therapeutics 7, 2367-2376 (2008).
12. Martinez, F.O. Analysis of gene expression and gene silencing in human macrophages. Current protocols in immunology / edited by John E. Coligan ... [et al.] Chapter 14, Unit 14 28 11-23 (2012).
13. Troegeler, A. et al. An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells and macrophages. Immunology and cell biology 92, 699-708 (2014).
14. Panganiban, R.A., Mungunsukh, O. & Day, R.M. X-irradiation induces ER stress, apoptosis, and senescence in pulmonary artery endothelial cells. International journal of radiation biology 89, 656-667 (2013).
15. Kim, K.S., Kim, J.E., Choi, K.J., Bae, S. & Kim, D.H. Characterization of DNA damage-induced cellular senescence by ionizing radiation in endothelial cells. International journal of radiation biology 90, 71-80 (2014).
16. Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J. & Toussaint, O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and *in vivo*. Nature protocols 4, 1798-1806 (2009).
17. Roper, R.L. Antigen presentation assays to investigate uncharacterized immunoregulatory genes. Methods in molecular biology 890, 259-271 (2012).
18. Santambrogio, L. et al. Extracellular antigen processing and presentation by immature dendritic cells. Proceedings of the National Academy of Sciences of the United States of America 96, 15056-15061 (1999).
19. Johansson, A.C., Steen, H., Ollinger, K. & Roberg, K. Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine. Cell death and differentiation 10, 1253-1259 (2003).
20. Kagedal, K., Johansson, U. & Ollinger, K. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15, 1592-1594 (2001).
21. Emert-Sedlak, L. et al. Involvement of cathepsin D in chemotherapy-induced cytochrome c release, caspase activation, and cell death. Molecular cancer therapeutics 4, 733-742 (2005).
22. Gerweck, L.E., Vijayappa, S., Kurimasa, A., Ogawa, K. & Chen, D.J. Tumor cell radiosensitivity is a major determinant of tumor response to radiation. Cancer research 66, 8352-8355 (2006).
23. Ogawa, K., Murayama, S. & Mori, M. Predicting the tumor response to radiotherapy using microarray analysis (Review). Oncology reports 18, 1243-1248 (2007).
24. Garcia-Barros, M. et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 300, 1155-1159 (2003).
25. Ji, X. et al. Cancer-associated fibroblasts from NSCLC promote the radioresistance in lung cancer cell lines. International journal of clinical and experimental medicine 8, 7002-7008 (2015).
26. Jiang, H. et al. Activated macrophages as a novel determinant of tumor cell radioresponse: the role of nitric oxide-mediated inhibition of cellular respiration and oxygen sparing. International journal of radiation oncology, biology, physics 76, 1520-1527 (2010).
27. Schneemann, M. & Schoeden, G. Macrophage biology and immunology: man is not a mouse. Journal of leukocyte biology 81, 579; discussion 580 (2007).

Annexes

Genetic characteristics of a selection of human CRC cell lines

Table 1 – Main genetic characteristics of a selection of human CRC cell lines. For each cell line, information regarding the number of mutations per Mb, the percentage (%) of non-diploid cells, which positively correlates with CIN[1], and the mutation status of some genes is indicated. Abbreviations: Mb - megabase pairs, S - stable, U - unstable, wt - wild-type gene, mut - mutated gene. Note: Cell lines with more than 25 mutations/Mb are considered hypermutated. [a] cell lines derived from the same primary tumour; [b] cell lines derived from primary tumour or metastasis from the same patient. Adapted[2] with permission from the American Association for Cancer Research (AACR). Table complemented with data from American Type Cell Culture (ATCC) website.

Organ source/ Cell Line	Derived from	Mutations per Mb	Non-diploid (%)	MSI status	Gene mutations in different signalling pathways								
					Wnt	MAPK		PI3K	TGF-β		p53		MMR
					APC	*KRAS*	*BRAF*	*PIK3CA*	*SMAD4*	*TGFBR2*	*TP53*	*ATM*	*MLH1*
Colon													
CACO2	primary tumour	9,58	92,7	S	mut	wt	wt	wt	mut	wt	mut	wt	wt
COLO205	metastasis	8,47	83,5	S	mut	wt	mut	wt	wt	wt	mut	wt	wt
DLD1[a]	primary tumour	260,69	2,8	U	mut	mut	wt	mut	wt	mut	wt	wt	mut
HCT116	primary tumour	95,68	6,8	U	wt	mut	wt	mut	wt	mut	wt	mut	wt
HCT15[a]	primary tumour	256,73	0,5	U	mut	mut	wt	mut	wt	mut	wt	mut	wt
HT29	primary tumour	17,03	75,6	S	mut	wt	mut	mut	mut	wt	mut	wt	wt
LOVO	metastasis	82,58	19,9	U	mut	mut	wt	wt	wt	mut	wt	wt	wt
RKO	primary tumour	161,68	11,0	U	wt	wt	mut	mut	wt	wt	wt	mut	mut
SW620[b]	metastasis	21,23	29,9	S	mut	mut	wt	wt	wt	wt	mut	wt	wt
SW480[b]	primary tumour	17,69	32,6	S	mut	mut	wt	wt	wt	wt	mut	wt	wt
Rectum													
SW1463	primary tumour	9,67	99,0	S	mut	mut	wt	wt	wt	wt	mut	wt	wt
SW837	primary tumour	8,38	83,0	S	mut	mut	wt	wt	wt	wt	mut	mut	wt

Rectal cancer treatment – clinical protocol

In order to obtain an overview of the current treatment options for primary rectal, particularly the relevance of radiotherapy in the management of this type of cancer, the following scheme was elaborated (Figure 1).

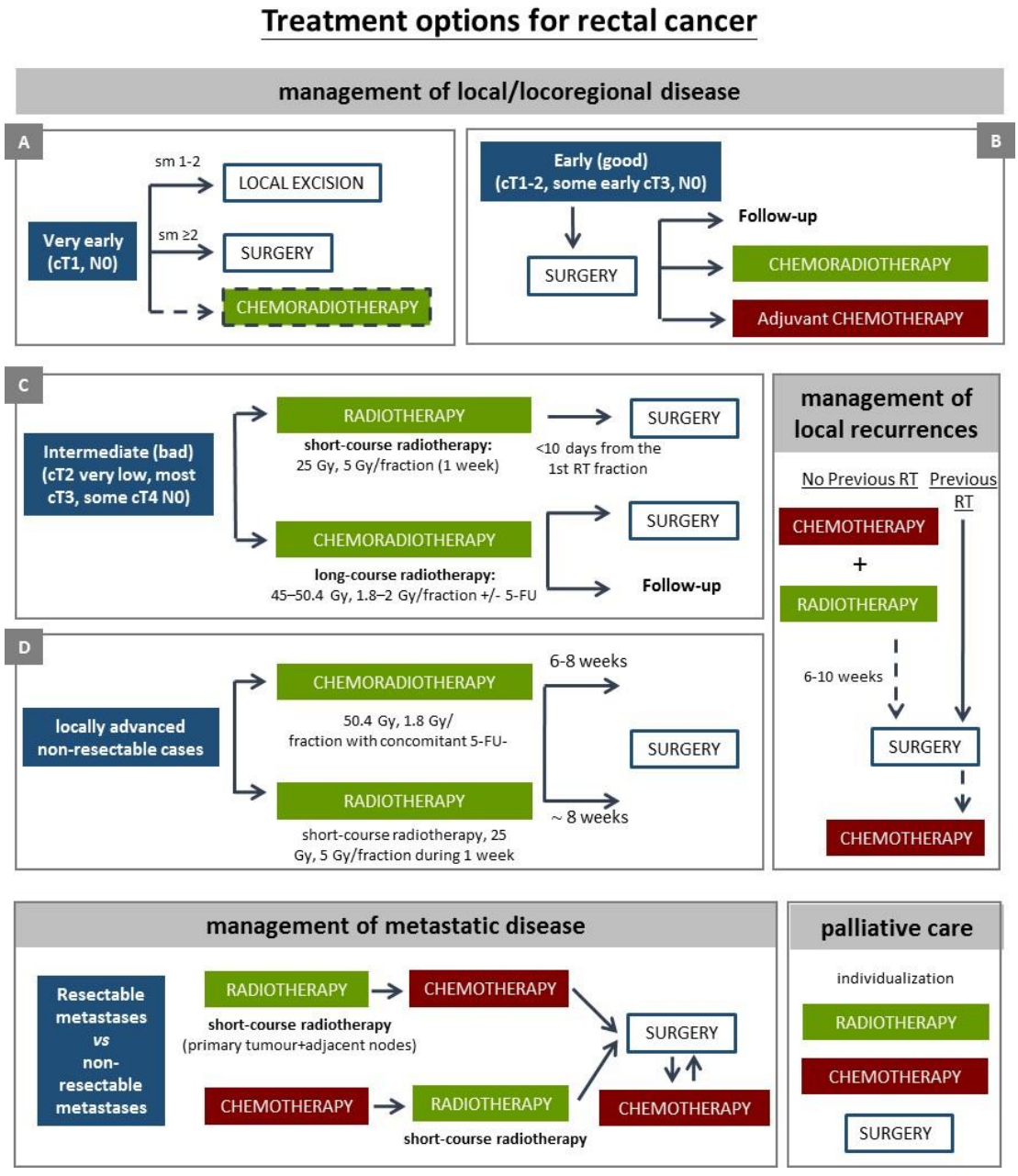


Figure 1 - Guidelines for the management of rectal cancer. This scheme was elaborate based on ESMO (European Society for Medical Oncology) and NCCN (National Comprehensive Cancer Network) Guidelines for rectal cancer treatment[3], in close collaboration with Dr Margarida Marques (CHSJ, Porto). Abbreviation: sm stands for the level of invasion into the submucosal layer for T1 stage: 1-upper third, 2-middle third, 3-lower third, which predicts the risk of lymph node metastases and thus the type of surgery.

From a practical point of view, locoregional rectal cancers could be divided into four groups: very early (some cT1), early (cT1-2, some cT3), intermediate (cT3- some cT4a) and locally advanced (cT3crm +, some cT4a, all cT4b). Rectal cancer treatment depends on the clinical stage of the tumour, prognostic markers as well as on the patient performance status. The guidelines for the management of locoregional as well as metastatic rectal cancer will be provided.

Treatment options for rectal cancer without metastases:

A) In very early cases, the removal of malignant polyps, frequently using transanal endoscopic microsurgery (TEM) is the appropriate choice. In case of incomplete resection with signs of vessel invasion or poor differentiation, immediate radical standard surgery (total mesorectal excision, TME) is recommended. In case of surgery contraindication, chemoradiotherapy (CRT) can be carried out, although this is a very rare situation; **B)** in early, favourable cases, a sharp radical dissection using the TME technique or partial mesorectal excision for tumours situated in the upper third of the rectum can be carried out. In case of poor prognostic signs (such as lymph node invasion) post-operative CRT or chemotherapy (CT) should be added; **C)** For intermediate cases, preoperative radiotherapy followed by TME is the recommended option, since it reduces local recurrence rates. In case of complete response after CRT, it is important to wait-and-see in high risk patients for surgery; **D)** in locally advanced cases, CRT followed by TME or a more extended surgery due to tumour overgrowth may be required. However, in very old patients (≥80–85 years) and in those that not fit for CRT (due to severe comorbidity), short-course radiotherapy with a delay to surgery is the best option.

Of note, pre-operative radiotherapy (45–50.4 Gy, 1.8 Gy/fraction, or alternatively 50 Gy, 2 Gy/fraction) is usually delivered concomitantly with radiosensitizer chemotherapy, which consists in the delivery of 5-FU, given either as bolus injections with leucovorin (at 6–10 times during the radiation) or as prolonged continuous infusion or oral capecitabine.

Treatment options for rectal cancer local recurrence

In case of recurrence, and if radiotherapy was not given in the primary situation, patients should receive preoperative radiotherapy with concomitant CT, followed by surgery 6–10 weeks after radiotherapy. When surgery is not possible, the patient may continue to receive CT, although the decision depends on each situation. In patients previously irradiated, surgery is the first option and CT may be considered after an evaluation. Reirradiation may also be an option, but this requires a carefully analysis of the doses previously given to make sure the healthy tissue can tolerate it.

Treatment options for rectal cancer with metastases

The treatment options for rectal cancer with metastases depend essentially on their resectability. If the patient can tolerate intensive treatment, short-course radiotherapy, followed by CT and surgery is the best option. Post-operative CT should also be considered, being recommended a total of 6 months of pre- and post-operative CT. Surgery to remove metastases may be preceded or followed by CT, usually after surgery for the primary tumour. Individualized therapy should be considered in case of primarily disseminated disease (synchronous metastases). Alternatively, the treatment may start with continuous CT until sufficient downstaging was observed, followed by short-course RT, if desired. Then liver/lung surgery and subsequent rectal cancer surgery with additional adjuvant chemotherapy, if considered of value, should be performed. If within the criteria, a very accurately type of radiotherapy, stereotactic body radiation therapy (SBRT), may also be performed.

Palliative care

Depending on the situation, RT, CT, surgery, or a combination of these approaches may be employed in palliative care. Palliative radiotherapy is delivered aiming to reduce symptoms, such as pain, and to control haemorrhagic situations, while surgery may be required to solve rectal occlusion and when the priority is to normalize the intestinal traffic, but only in patients with good performance status. In case of bad performance status, a colostomy or prosthesis will temporarily solve the situation.

References

1. Miyazaki, M. et al. The relationship of DNA ploidy to chromosomal instability in primary human colorectal cancers. Cancer research 59, 5283-5285 (1999).
2. Mouradov, D. et al. Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. Cancer research 74, 3238-3247 (2014).
3. Glimelius, B., Tiret, E., Cervantes, A., Arnold, D. & Group, E.G.W. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology: official journal of the European Society for Medical Oncology / ESMO 24 Suppl 6, vi81-88 (2013).