UiT THE ARCTIC UNIVERSITY OF NORWAY

Molecular Inflammation Research Group
Faculty of Health Sciences
UiT-The Arctic University of Norway

“EFFECT OF IRRADIATION ON THE IMMUNOREGULATORY FUNCTIONS EXERTED BY TUMOUR FIBROBLASTS ON MACROPHAGES”

Muhammad Asad Akhtar

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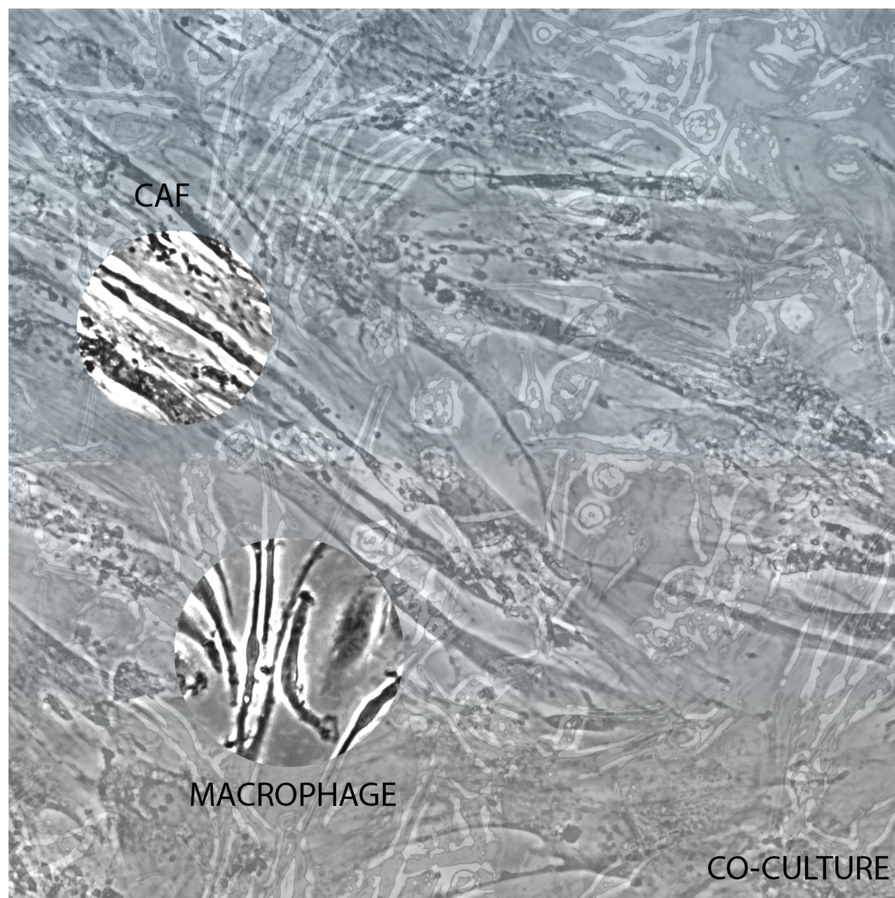

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Summary

Background/aim of the study: Carcinoma-associated fibroblasts (CAFs) represents a heterogeneous population of cells and are considered one of the dominant stromal component of solid tumours, performing a crucial role in tumour proliferation and metastasis. The main objective of this study was to investigate the immunoregulatory features of CAFs isolated from non-small cell lung carcinomas on macrophages and the potential effects of ionizing radiation on observed effects.

Methods: The study comprise primary cultures of CAFs isolated from freshly resected NSCLC (Non Small Lung Cancer) tumours (n=8) and monocyte-derived macrophages prepared from peripheral blood of healthy donors. The experimental settings included both co-cultures and incubations of macrophages with CAF-conditioned medium. Moreover, CAF-mediated effects were studied in both uncommitted and M1-polarized macrophages. Functional assays to study macrophage polarisation included expression of cell surface markers by flow cytometry, production of nitric oxide by flow cytometry and secretion of inflammatory cytokines by ELISA.

Results: All functional assays illustrated that CAFs both in co-culture and by conditioned medium, promote changes on uncommitted macrophages (M0) that harmonize with both M1 and M2 phenotypes. CAFs, both in co-culture and by conditioned medium, could inhibit some of the pro-inflammatory features of M1 macrophages as demonstrated by strong inhibition of nitric oxide production, strong inhibition of proinflammatory cytokines secretion and a reduction of some M1 surface markers. Importantly, radiation given at high dose (1x18 Gy) or in fractioned regimens (3x6 Gy) is not able to modify substantially the immunoregulatory features exerted by CAFs over macrophages *in vitro*. Last, protein expression analyses in CAF supernatants show that both irradiated and non-irradiated CAFs produced approximately the same levels of cytokines.

Conclusion: This study display that CAFs-derived soluble factors mediate measurable changes on uncomitted macrophages (M0) and down-regulate pro-inflammatory features of M1 polarized macrophages, even though the soluble factors accountable for this shift remains unknown. On the other hand, this study also illustrates that low dose fractioned radiotherapy and single high dose radiotherapy do not curtail the immunosuppressive effect of CAFs.

Abbreviations

NSCLC: Non-Small Cell Lung Cancer

CT: Computerized Cromatography

MRI: Magnetic Resonance Imaging

PET: Positron Emission Tomography

SABR: Stereotactic Ablative Radiotherapy

HD-RT: High-Dose Radiotherapy

CAFs: Carcinoma-Associated Fibroblasts

BMDC: Bone Marrow Derived Mesenchymal Cells

TAM: Tumor Associated Macrophages

ECM: Extracellular Matrix

EMT: Epithelial Mesenchymal Transdifferentiation

α-SMA: α-Smooth Muscle Actin

FAP: Fibroblasts Activation Protein

FSP-1: Fibroblast-Specific Protein-1

PDGF: Platelet-Derived Growth Factor

MMPs: Matrix degrading Metalloproteinase

uPA: urokinase Plasminogen Activator

SDF1: Stromal-Derived Factor 1

IL: Interleukin

VEGF: Vascular Endothelial Growth Factor

HGF: Hepatocyte Growth Factor

EGF: Epidermal Growth Factor

TGF-β: Transforming Growth Factor-β

FGF2: Fibroblasts Growth Factor 2

EPC: Epidermal Progenitor Cell

TN-C: Tenascin-C

MDSC: Myeloid-Derived Suppressor Cell

NK: Natural Killer Cell

DC: Dendritic Cell

PGE2: Prostaglandin E2

IDO: Indoleamine 2,3-Dioxygenase

PD: Programmed Death

CTL: Cytotoxic T lymphocytes

Th: T helper

IFN-γ: Interferon-γ

M1: Classically activated Macrophage

Treg: T regulatory cells

M2: Alternatively activated Macrophage

SOCS-1: Suppressor of Cytokine Signaling

SHH: Sonic Hedgehog

M-CSF: Macrophage-Colony Stimulating Factor

TLR: Toll-Like Receptor

TSP: Thrombospondin

ELCAP: Early Lung Cancer Action Project

IGRT: Image Guided Radiotherapy

ICD: Immunogenic Cell Death

DAMPs: Damage-Associated Molecular Patterns

HMGB1: High-Mobility Group Protein-1

CRT: Calreticulin

HSP: Heat Shock Proteins

VCAM-1: Vascular Cell Adhesion Molecule-1

YAP-1: Yes-associated protein 1

1 Introduction

1.1 General principles on tumour immunology

1.1.1 The immune system

The immune system can protect us against infectious organisms and transformed cells, including tumour cells. It is broadly divided into two categories: innate immunity and adaptive immunity. Innate immunity comprises cellular, biochemical and physical structural protective mechanisms that provide the first line of defence against any infectious agents or transformed cells in a non-specific manner. It includes the natural killer cells, macrophages, granulocytes, dendritic cells mast cells, cdT cells and natural killer T cells. While adaptive immunity provides a specific immunity and composed of T-lymphocytes, B-lymphocytes and their humoral mediators including cytokines and antibodies [1].

In the context of tumours, immunology portrays a relationship between the cells of the immune system with tumour cells. Tumour cells are mutationally corrupted, and their further development and progression depend upon the interaction between immune cell and tumours cells. Within cancer, the immune system acts in three different ways [1]. First, the immune system can defend the host against virus-induced tumours by suppressing or eliminating the viral infection. Second, the immune system can timely resolve the inflammation to hinder the development of inflammatory condition, which assists in tumorigenesis. Thirdly, the immune system can specificallly recognise and eliminate the tumour cells by interacting with tumour-specific antigens or the molecules induced by cellular stress (immunosurveillance).

1.1.2 Cancer Immunoediting: from immunosurveillance to immune escape

Paul Ehrlich first emphasised the significance of the immune system against tumours and this concept became formally presented as cancer immunosurveillance by Burnet and Thomas [2]. After that, there has been a significant number of studies observing the increased frequency of tumour in immune-deficient individuals, both in mice and humans [3]. Furthermore, people who were treated with immunosuppressive drugs following the transplantation and also HIV-positive individuals, demonstrate a high rate of tumour formation [4]. These observations collectively, confirms the idea of immunosurveillance. However, these concepts confronted a few restrictions because malignancy also happens in patients which have an effective immune system. Moreover, *Prehn et al.* endorsed that the immune system can indeed propel tumour development [5]. This dual

part of the immune system is explained through the concept of immunoediting by Robert Schreiber [3].

1.1.2.1 Three phases of cancer Immunoediting

The immunoediting theory contains three 'Es.' The first "E" refers to **elimination**, which indicates the immunosurveillance capacity of the host immune system to eliminate tumour cells in incipient tumours (*Figure 1.1*) [3]. It can be called complete elimination when all the tumour cells are successfully eliminated, or incomplete when some of the tumour cells survive to the attack. According to immunoediting theory, incomplete elimination is a short-term of dynamic equilibrium between the immune system and developing a tumour.

The second phase is called the **equilibrium** phase, in which the host immune system and tumour variant cells that have escaped from the first phase enter into a dynamic equilibrium (*Figure 1.1*).

In that phase, the immune system exerts a continuous immunological pressure on tumours sufficient to limit their growth, but not entirely eliminate a tumour bed carrying many mutated cells. Albeit most of the primitive tumour cell variants are eliminated, new variations may emerge carrying different mutations that provide them with higher resistance to immune attack [3].

Furthermore, the third E refers to **escape** and is the last step in which the immune system is unable to restrict tumour growth (*Figure 1.1*). Along these lines, tumour cells develop new strains which evade the immune system and keep on flourishing relentlessly *(Figure 1.1*)[3]. This capacity of tumour cells is considered as a 7th hallmark of cancer [6]. The third phase of the immunoediting process is also termed immunoevasion. Nowadays, much research is directed to comprehend the different process of immunoevasion for the advancement of better immune-therapeutic techniques to combat cancer [7].

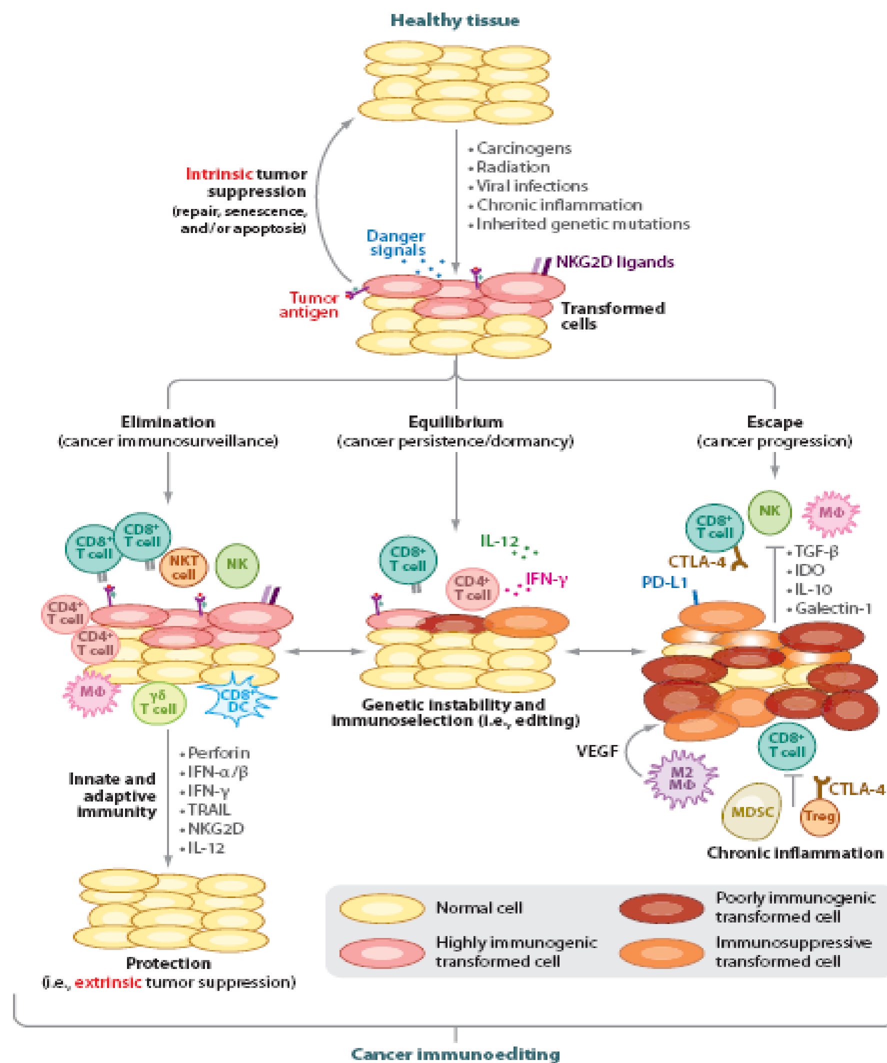


Figure 1.1: Three phases of cancer Immunoediting: *Three phases of cancer immunoediting which may occur separately or collectively. One normal cell changed into cancer under multiple factors. First "E" explains the ctive role of immunity to protect against cancer. The second E represent the equilibrium state. The third E indicate Escape in which tumor adopt new variants which successfully evade the immune system and become clinically detectable. Adapted from Vesely et al Annual review of Immunology 29, 235-271 (2011).[8]*

1.1.3 Mechanism of Cancer Immune evasion

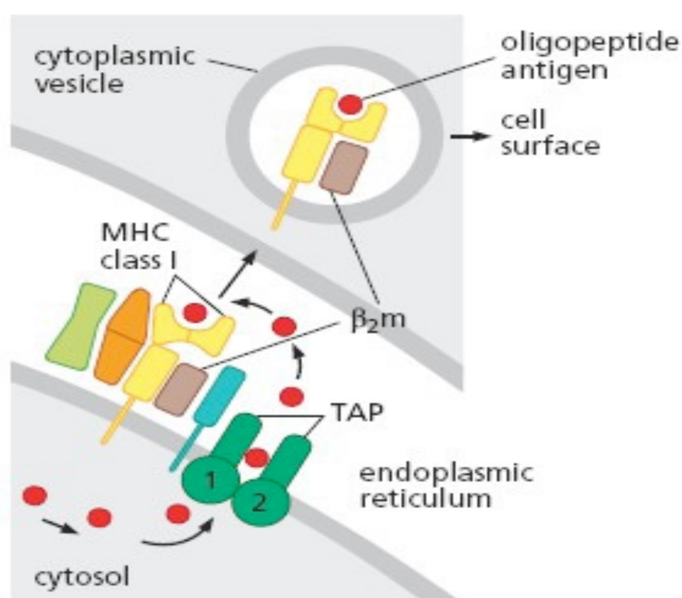
1.1.3.1 Immunoselection of poorly immunogenic tumor cells

As tumour progress, tumour cells continuously modified themselves at the genetic and epigenetic level to dodge the immune system. Furthermore, the immune system implies a continuous immunological pressure which facilitates the Darwinian selection of most suitable tumours strain to survive and multiply in the immunocompetent host and their offspring turn into the dominant population in atumour [9].

1.1.3.2 Reduction of antigen presentation

Tumour cells being innate in origin reflect self-antigens on their surface to which immune cells have tolerated during development [4]. However, due to the continuous mutational changes experienced by cancer cells after uncontrolled cell divisions, tumour-specific neoantigens may appear in tumour cells [10]. The immune system can identify them as a threat and mount a response against it [10]. However, tumour cells hide their identity, by changing its antigenicity, to avoid the surveillance of immune cells. This is one of the well-recognised and remote concepts in tumour immune evasion. One of the proposed strategies is promoter methylation to repress the expression of a specific tumour antigen-encoding genes [7]. As an alternative mechanism, tumour cells can shed their antigens temporarily by endocytosis or permanently into the circulation [3].

However, the down-regulation of MHC class I molecules in tumours, may alert NK cells, which are constantly screening the body's tissues for cells which have lost the appropriate number of those receptors from their surface [11]. As an immune-evasive strategy, tumour avoid the attack by NK cells by repressing one of the six key MHC-I molecule [12]. On the other hand, some tumours utilized another route, by downregulating the antigen processing machinery especially MHC-I gene transcription [13](13). Sometimes, tumour cells also used the post-translational mechanism for repressing the necessary component of the MHC-I complex, for example, beta-2 microglobulin and TAP protein, which has the essential role in antigen presentation *(Figure 1.2)* [14]. Deregulation of MHC-II molecule of antigen presenting cell is also observed, mostly the class-II trans-activator or the different portion of MHC-II pathways like HLA-DM and HLA-DO are affected[14].



FFigure 1.2 *This figure displays the involvement of heterodimer TAP1&2 and B-2 macroglobulin wit MHC class 1 molecule in antigen presentation on cell surface. Failure of any Component preclude the antigen presentation. TAP-Transporter associated with antigen processing, MHC-Major histocompatibility complex. Adapted from Weinberg, R. A. The Biology of Cancer. (Gerald Science, 2013)[12]*

1.1.3.3 Activation of anti-apoptotic mechanisms

Tumour cells implied numerous strategies to avoid apoptosis, as it is one of the hallmarks of cancer [6]. For that purpose, tumour manipulate their biochemical profile, by increasing the level of anti-apoptotic proteins (c-FLIP, Bcl-xL, Mcl-1) and cytokines (IL-4, IL-10, prostaglandin E2 and TGF-b), in response to immune-induced cytotoxicity, hypoxia, loss of anchorage, etc [15, 16]. Tumour cells may also increase the expression of inhibitors of perforin/granzyme pathway to avoid CD8+ T-lymphocyte mediated cytotoxicity, as it was observed in a lung cancer study by Soriano et al [17].

As an evasive strategy, some type of cancer cell start producing a stress-related protein (MICA, MICB, ULBP4 and so on), around its surroundings as a trap. These proteins bind with NKGD2 receptors on NK cells, which ultimately lead to endocytosis and degradation of NK cells [18]. One study in colon cancer indicated the release of DC3 receptor interact with Fas ligand to dodge the Fas-FasL induced apoptosis in immune cells [19].

Moreover, it has been observed that tumour cells may carry antibodies on their surface, and these antibodies carrying cells should lead to apoptosis by complement dependent cytotoxicity. However, cancer cells increase the magnitude of membrane-bound complementary regulatory proteins (m-CRPs) (CD-46, CD-55, and CD-59) in their surroundings to evade it [20]. Tumour cells also decrease the expression of death receptor CD95 through deprivation or mutation of wild-type CD-95 as in various cases of leukemias, hepatocellular tumours, and its related with the worst outcome [21].

1.1.3.4 Metabolic Reprogramming

A significant proportion of tumour cells modify their metabolism to generate ATP from glycolysis instead of mitochondrial respiration, even in the presence of oxygen (Warburg effect) [22]. This modification of metabolism is critical to deal with the high energy need of the malignancy and for immune evasion as well. Regarding ATP production, tumour glycolysis prompt 18 times less ATP generation than oxidative phosphorylation [23]. Tumour cells preferred along these lines by increasing the rate of glycolysis up to 100 times [22] and used other biological molecules (NADPH, ribose five phosphates) for their expansions [23]. These metabolic changes may reshape the tumour microenvironment, as metabolic sub-products of glycolysis, such as, a lactic acid may create an acidic PH intratumorally which can be toxic to normal cells as shown in *Figure 1.3*. The elevated levels of lactate in the tumour microenvironment influence the efficiency and multiplication of T-cells, NK-cells [24], maturation of dendritic cells [25], degranulation of perforin, and effectiveness of chemokine in tumour favour [26].

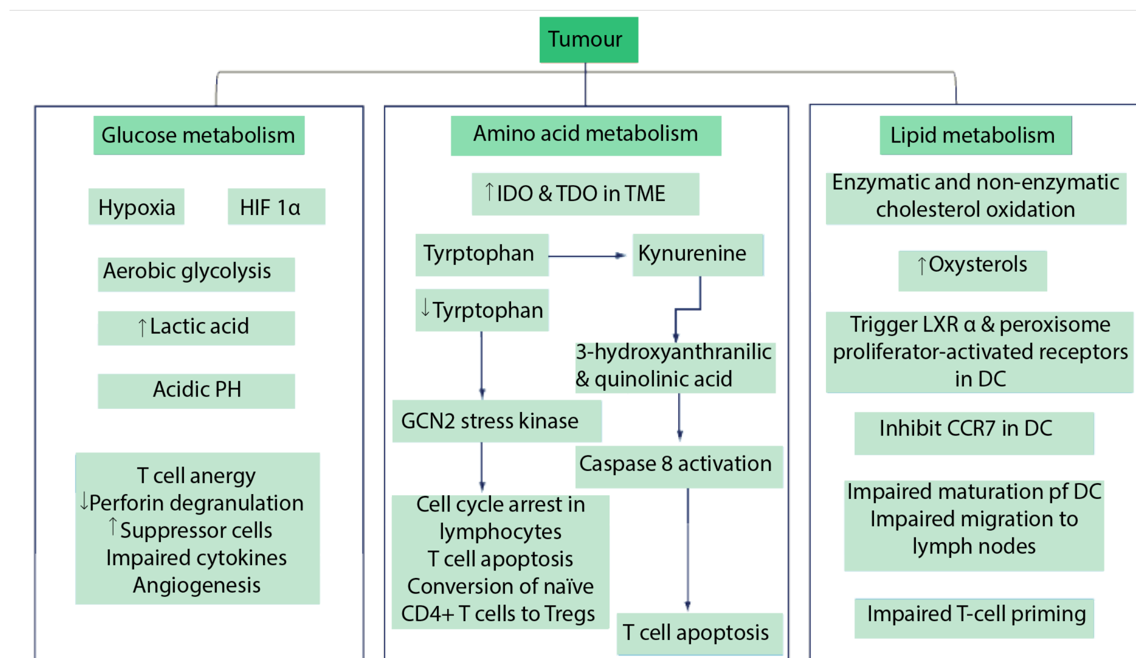


Figure.1.3:Modification of cancer cell metabolism and immunoevasion. *Tumour metabolize glucose to generate lactic acid by aerobic glycolysis which leads to acidic Ph n TME and contribute in the immunosuppressive environment. IDO leads to speed up the metabolism of tryptophan which ultimately favours T cell apoptosis. While high level of LXR @ in dendritic cell affect its maturation and immune role in the tumour. DC: Dendritic cell; IDO: Indoleamine 2,3-dioxygenase; MDSC: Myeloid-derived suppressor cell; TDO: Tryptophan 2,3-dioxygenase; TME: Tumour microenvironment. Retrieved and modified from Bhstis et al Expert review of clinical immunology* ***10****, 41-62 (2014).[27]*

The modified metabolism in tumour cells leads to the aggregation of indoleamine 2,3-dioxygenase (IDO), and tryptophan 2,3-dioxygenase (TDO), in amino acid (tryptophan) metabolism (*Figure 1.3*). These provoke the inactivity of T-cell immune response, enrolment of T-reg cells and modulation of cytokine (TGF-β, IL-6, and IL-10), through GCN-2 (general control non-depressible 2) stress kinase [28, 29].

Moreover, the modified metabolism and unlimited growth, lead to the condition of hypoxia in their microenvironment where hypoxia inducible component (HIF-a) expressed, which encourage the angiogenesis, and extension of immunosuppressive cells which assist in immunoevasion [30]. There is plenty of evidence in favour of that metabolic change which plays a significant role in developing an immunosuppressive environment, which ultimately supports immune evasion.

1.1.3.5 Expression of co-regulatory receptors

Tumour cell introduces numerous strategies to create a microenvironment in which the action of immune cells is compromised. There are several receptors present on immune cells which can

control the overreaction and self-attack. Many of these receptors, like CTLA-4, PD-1, ICOS (inducible T-cell co-stimulator), BTLA (B and T lymphocyte attenuator) and Fas death receptor are present on T-cells and show inhibitory control upon stimulation with the related ligand on tumour cells or APC (antigen presenting cell) [31] as shown in *Figure 1.4*.

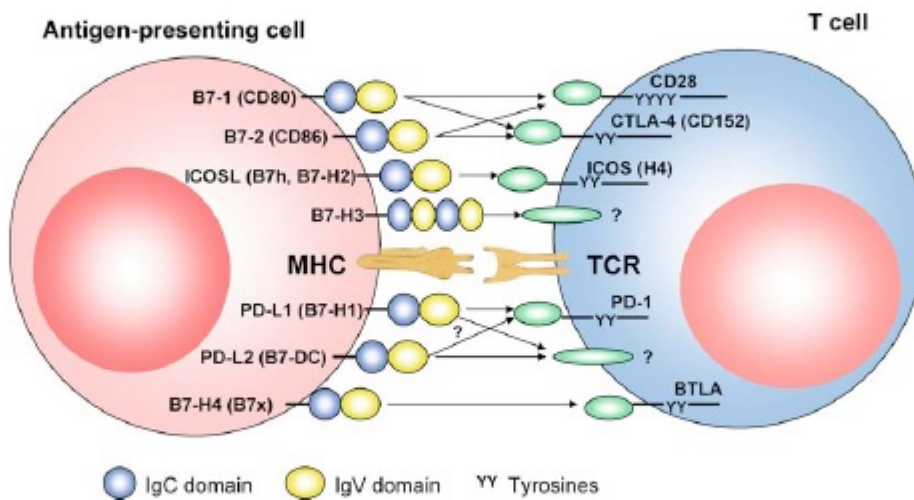


Figure 1.4: Co-regulatory Receptors: *It displays the interaction between the molecule of B7 superfamily molding TCR signaling. CD-28 and CTLA-4 communication with respective B7 ligand provide a negative signal upon T-lymphocyte enactment. ICOS bind with ICOS-L. PDL and PD interaction negatively regulates T&B cell activity (36). Retrieved and modified from Blank et al Cancer research* ***64****, 1140-1145 (2004)[32]*

Tumour cell utilized these ligands to suppress the attack of tumour-specific T-cell and IL-10 and IFN-γ secretion. Beyond this, tumour cells also take control of that type of killing by getting the capacity of producing and directing the Fas ligand to the Fas death receptor displayed by numerous lymphocytes and killed them [19, 33]. In this way by killing immune cells, tumoruss keep up a safe zone for its growth.

1.1.3.6 Recruitment of immunosuppressive Immune cells

As another immunoevasive strategy, tumour cells promote the recruitment of immunosuppressive cells into tumors, such as regulatory T-lymphocytes (from 5 % to 30) or immunosuppressive myeloid cells[6]. Tumours release various cytokines (TGF-β), chemokines (CCL22, CCL28) and HIF-α that promotes the recruitment of T-regulatory cells as shown in *Figure 1.5* [34].

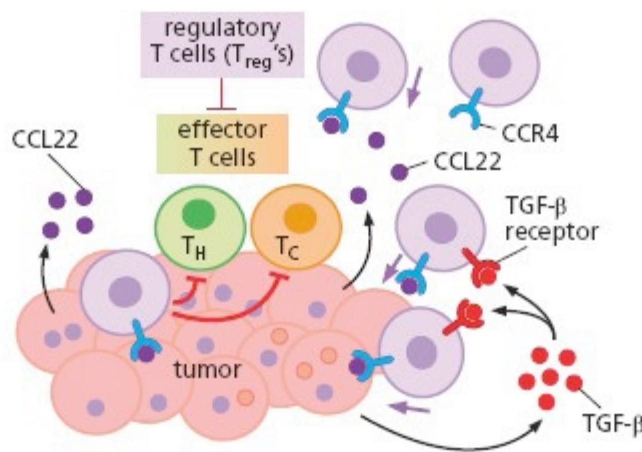


Figure 1.5: T-reg cell and immunoevasion *Regulatory T-cell and Tumour immune evasion. Tumour cells release the TGF-β and CCL22 chemokines, later binds the CCR4 chemokines receptor of the Treg and in this way, attracts them into the tumour. Treg cells have the inhibitory effect on cytotoxic and helper T-cells (12) Retrieved and modified from Weinberg, R. A. The Biology of Cancer. (Gerald Science, 2013)[12].*

These T-regulatory lymphocytes can suppress and kill the helper T-cell and cytotoxic T-cell that recognise the same antigen as the T-regulatory cell and involve in T-cell tolerance [35]. In this way, by inhibiting the role of cytotoxic T cell and suppressing the action of helper T-cell, which can initiate the humoral and cellular immune response, T-regulatory cells assist in immunoevasion.

Tumour cells also increase the myeloid-derived suppressive cells (MDSCs) as an immunoevasion approach. These cells facilitate a tumour immune evasion by employing the L-arginine (dependent & independent) procedure[36]. MDSCs secrete two enzymes, Arginase-1(AGR1) enzyme transform L-arginine into urea, and inducible nitric oxide synthase-2(iNOS2) converts L-arginine into nitric oxide (NO) and L-citrulline [37]. The elevated level of NO and superoxide ion induce the apoptosis of T-lymphocyte, while the production of peroxynitrites disturbs the peptide binding of T-lymphocyte and make them insensitive to antigen-specific immune stimuli [38, 39]. The L-arginine independent procedure leads to CD-62L downmodulation, cysteine reduction, ROS and TGF-β production which hs a role in immunoevasion [40]. MDSc also secret VEGF, fibroblast growth factor, HIF-1, TGF-β and MMP-9 which may directly inhibit immune attack [41].

1.1.3.7 Development of mechanical barriers

Tumour microenvironment (TME) consists of cells from endothelial, mesenchymal, and hematopoietic origin embedded in a complex extracellular matrix (ECM). These various components and their secretions play a vital role to suppress the immune network and assist in tumour growth. **ECM** (collagen, laminin, thrombospondin and fibronectin), have a role in immunoevasion by interfering with trafficking of immune cells [42]. Such as thrombospondin have a role in diminishing the T-lymphocyte efficacy by maintaining the dendritic cell (DC) in an immature state [43], and by suppressing IL-12 secretion in tumour microenvironment [44].

As an immune evasive strategy, tumour also increases the percentage of pro-tumour macrophages (TAM) and CAFs in their surroundings. In the following sections, It will be describe the role of CAFs and macrophages in tumour development.

1.2 Cancer Associated Fibroblasts

1.2.1 The heterogenic nature of CAFs

Fibroblasts are one of the most abundant and multipurpose components of the tumour stroma, and their existence in higher amounts in a solid tumour is connected with poor prognosis [45]. It has been reported in many studies that tumour cells release various growth factors (TGF-β, PDGF, EGF, FGF) to recruit and activate tissue resident fibroblasts into the stroma [46, 47]. Several other local sources of CAFs have also been reported, like epithelial cells, endothelial cells, pericytes, adipose tissue, stellate cells, bone marrow cells which can differentiate into CAFs under the influence of tumour cells [48] *(Figure 1.6)*.

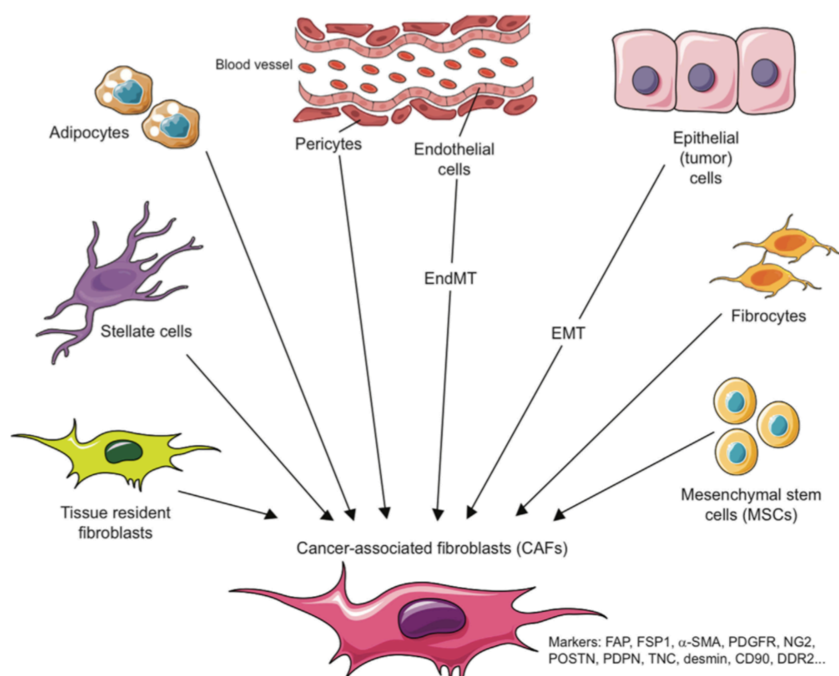


Figure 1.6: *CAFs originate from different cell population via different mechanisms (55). Ziani et al,2018, Adapted Frontiers in immunology* ***9****, 414 (2018).*

It has been reported in various studies that CAFs may originate from tumour cells by epithelial to mesenchymal trans-differentiation (EMT) [42]. However, this seems unlikely as CAFs are not tumorigenic themselves, and it is still debatable whether CAFs carry mutations in their genetic profile or not [49, 50]. The diverse sources of CAFs explain the heterogeneity of CAFs and make them hard to recognise from other mesenchymal cell types in TME *(Figure 1.6)*.

For their identification, CAFs express different markers that are lower or not expressed by normal fibroblast, such as α-smooth muscle actin (α-SMA), fibroblasts activation protein (FAP), fibroblast-specific protein-1 (FSP-1), platelet-derived growth factor receptors (PDGFR), α/β, neuron-glial antigen-2 (NG2), periostin (POSTN), podoplanin (PDPN), tenascin-C (TNC), desmin, CD90/THY1, or discoidin domain- containing receptor 2 (DDR2) [50, 51]. In the same way, Activated fibroblasts may not express all these markers simultaneously, probably showing the heterogenic nature of CAFs in TME´ and also their variable function in relation to TME [50, 51].

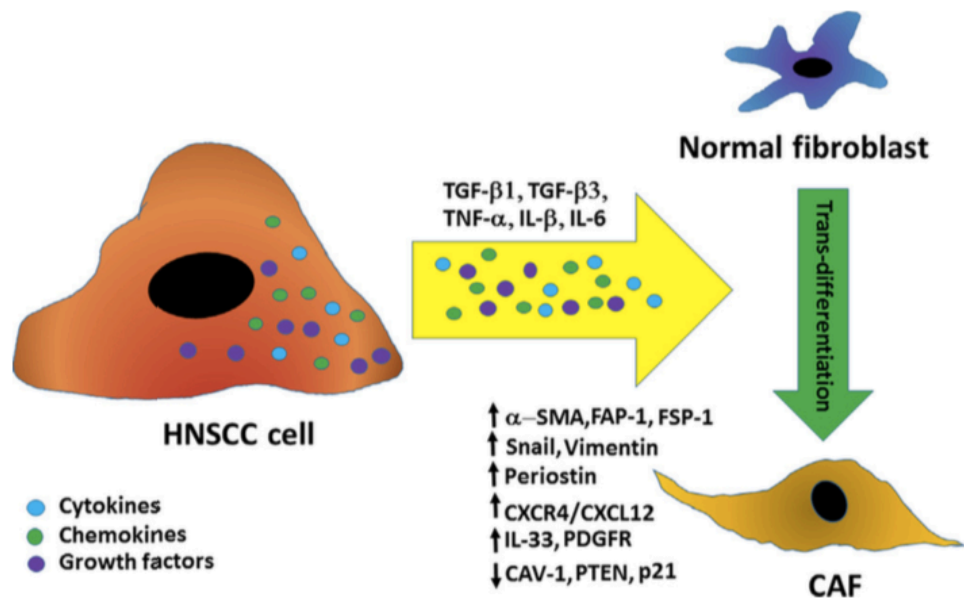


Figure.1.7: *Schematic description of normal fibroblast into CAFs. Tumour cells derived cytokines, chemokines and growth factors mediated paracrine trans-differentiation of resident fibroblasts into CAFs. There is an upregulation of Vimentin, a-SMA, FAP-1, FSP-1, Snail, CXCR4/ CXCL12, IL-33 and PDGFR surface markers and downregulation of CAV-1, PTEN and p21 in CAFs (Retrieved and modified from Utispan et al, 59, 23-30 (2017).[52]*

1.2.1.1 Tissue Resident Fibroblasts versus CAFs

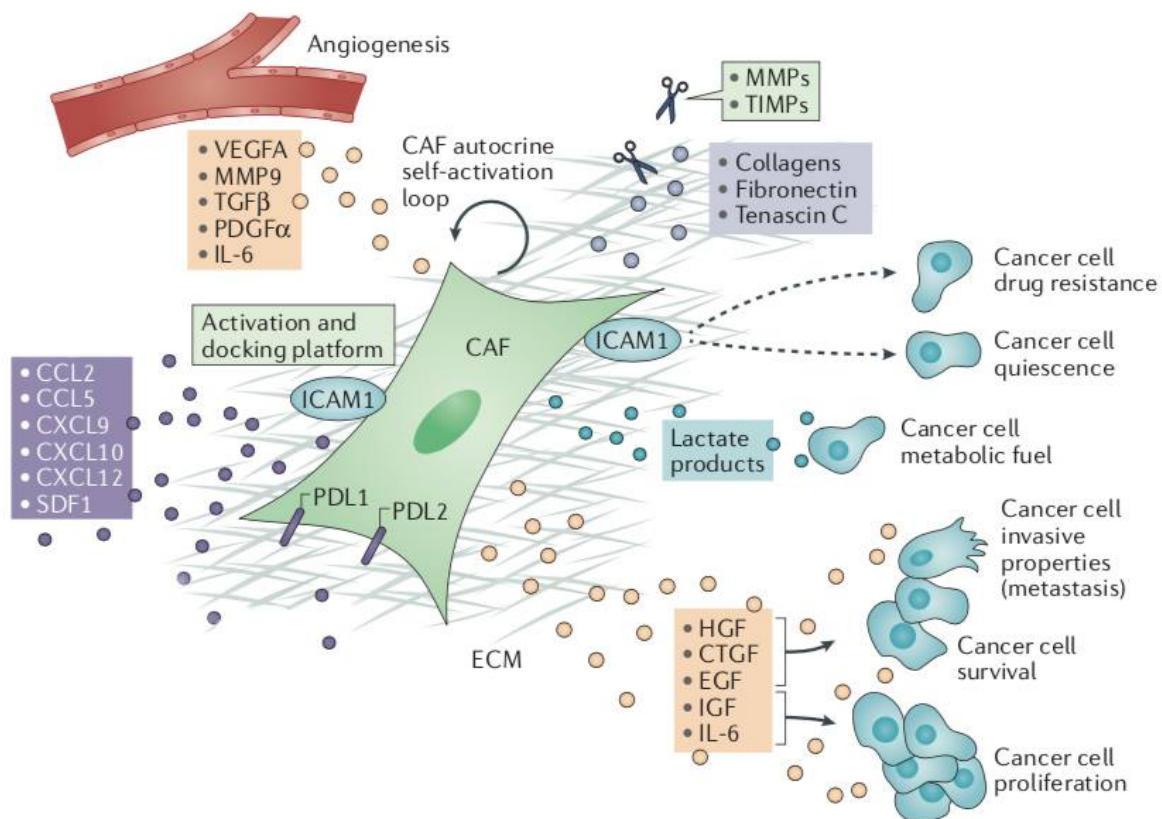
Fibroblasts have been characterised by their typically spindle-shaped morphology, which is present in a quiescent state with minimal metabolic and transcriptional activity. In healthy tissue, fibroblasts keep homeostasis and integrity of the connective tissue by releasing the extracellular matrix proteins (ECM) like integrin, collagen and fibronectin [53]. Fibroblasts are also a source of matrix-degrading proteases, such as (MMPs) which is critical for ECM remodelling [54]. In the case of wound healing or inflammation, fibroblasts become transformed into activated myofibroblast and increase the synthesis of ECM, producing chemokines, cytokines, enlisting immunocytes and applying mechanical forces to remodel the tissue structure [55].

In tumour microenvironment, activated fibroblasts or CAFs attain a higher degree of contractile potential that is related with their ability to adjust the tissue architecture, obtains a proliferation and migration potential and turns out to be transcriptionally active to secrete various cytokines, chemokines and ECM proteins [56]. This capacity of fibroblasts to become activated was first recognised in wound healing [57] and along these lines in many pathological conditions, such as in acute or chronic inflammation[56]. Likewise, in the case of solid cancers, this tissue repair process would go on and never return to the steady state, which is why cancers are considered as "wounds that never heal"[58].

1.2.2 CAFs role in Tumour Progression

Activated fibroblasts collected from human tumour stroma display specific characteristics compared to fibroblasts isolated from normal organs [59]. The capability of CAFs to assist in tumorigenesis is somehow dependent on their capability to prompt angiogenesis. CAFs release a high level of stromal-cell-derived factor-1 (SDF-1/CXCL12) and recruit bone-marrow-derived endothelial cells to assist in angiogenesis[60]. Numerous fibroblast activation ligands, such as TGF-β, bone morphogenic proteins (BMPs), platelet-derived growth factors (PDGFs), epidermal growth factors (EGFs), Fibroblast growth factors (FGFs) and sonic hedgehog (SHH) have a pro-tumorigenic role *(Figure 1.8)*. The elevation of heat shock factor 1 (HSF1) in CAFs enhances the HSF-1 a dependant tumour promoting mechanism in tumour cells, assisting a pro-tumour effect of the TME [61]. Similarly, Yes-associated protein-1 (YAP1) stimulation in CAFs increases ECM hardening and tumour cell infiltration [62]. De-regulation of p53 and Notch signalling pathways in CAFs assist their development [63]. CAFs secrete multiple matrix degrading protein (MMP-1, -3), which assist in motility and invasion of the tumour cell, as well as help in epithelial to mesenchymal transition (EMT) [64, 65].

Additionally, fibroblast-derived exosomes also support tumorigenesis by regulating the fibroblast function, metabolic reprogramming and induction of cancer stem cell characteristics [64, 65]. ECM remodelling by fibroblasts can also contribute to the production and retention of cancer stem cell position. Fibroblast could be educated by cancer stem cells to prompt an environment that could support cancer stem cell retention. Cancer stem cells are a potent stimulator of fibroblasts by thrombospondin-2 (THBS2) manifestation, advancing malignancy in lung cancer[66]. Paracrine communication between cancer stem cells and fibroblast-derived insulin growth factor-II (IGF2) and IGF-1 receptor (IGF1R) signalling in cancer stem cells, prompting Nanog manifestation and stemness-like attributes in tumour cells [42].



Figures 1.8: *The CAF assist cancer through angiogenesis, remodelling ECM and involve other secretions for their growth, survival and resistant therapy. The extracellular matrix (ECM), together with cellular components of the tumor microenvironment (TME), are actively remodelled and reprogrammed by cancer-associated fibroblasts (CAFs). ICAM1displayed on CAFs and served a s a site for immune cells functioning, while PDL1 &2 involved in immunosuppression. (Intercellular adhesion molecule-1 ICAM1, programmed cell death protein 1 ligand 1 (PDL1) retrieved and modified from Kalluri ER, et al, Nature Review Cancer (2016)[42].*

1.2.2.1 CAFs role in Metastasis

Metastasis is the ability of tumour cells to populate distant organs and establish a secondary tumour. In order to develop metastatic capabilities, tumour cells have to obtain motile features via EMT. In this way, tumour cells lose their cell-to-cell adherence and obtain a mesenchymal phenotype with an irregular mobile and migratory capability, allowing them to attack the encompassing stroma and ultimately intravasate the circulatory system.

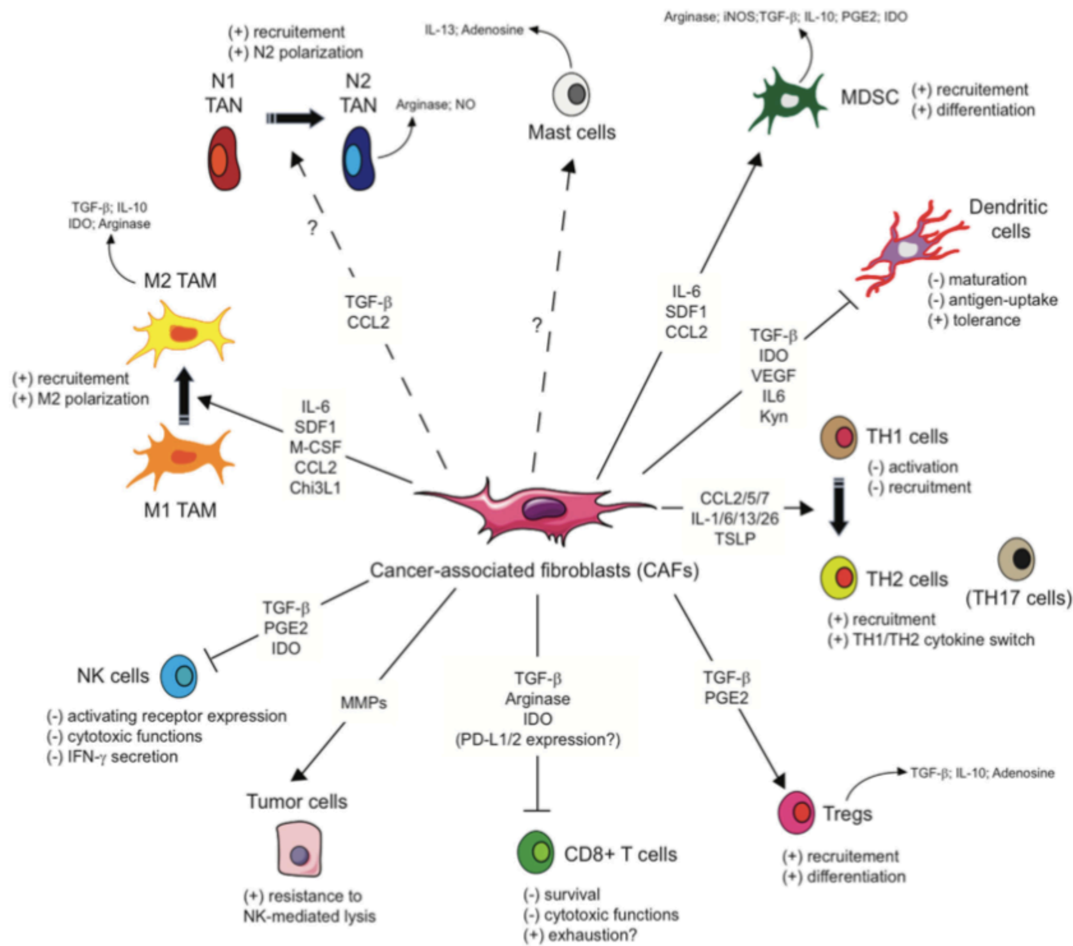
This procedure could be stimulated by CAFs via the production of HGF and GF-β [67]. CAFs could assist tumour cell invasion and proliferation by producing ECM-degrading MMPs. In this way, CAFs influence ECM density at the primary tumour site and may create pathways in the ECM to intensify the invasiveness of tumour cells[68]. This is the outcome of the release of matrix-

associated growth factors, for example, VEGF, which, at the same time, induces tumor angiogenesis [69]. For example, one of the investigations demonstrated that CAFs liberated Tenascin-C (TN-C) [70] upregulate the NOTCH and WNT signalling pathway in tumour cells, which instigate cell migration and metastatic ability as displayed in *Figure 1.8*. TN-C also contributes in tumor angiogenesis [71]. In addition to the well-defined role of fibroblasts in metastasis by supporting EMT, it is also reported that CAFs can separate from a primary tumour along with malignant cells and enter into blood circulation to protect the cancer cells. Along these lines, CAFs also contribute to create an appropriate microenvironment at the secondary site [72]. Another investigation revealed that FSP-1 positive CAFs improve tumour metastasis by advancing an inflammatory environment [73]. All the referred documentation confirms the pro-invasive and pro-metastatic function of CAFs and defines its role as a positive regulator of tumour growth and progression.

1.2.3 CAFs role in antitumour immune response

CAFs exert multiple immunomodulatory functions around tumours. CAFs can attain a secretory phenotype, facilitating the production of ECM protein, the manifestation of ECM remodelling enzyme and the secretion of plenty of cytokines and chemokines. A recent study supports the assumption that CAFs' secretion may regulate the immune cell recruitment and function in tumour mass [42]. In this respect, CAFs can modify the anti-tumour immune response and facilitate tumour progression *(Figure 1.8)*. However, these investigations are limited to *in vitro* studies only [42].

In general, CAFs are considered to provide an immunosuppressive TME [74]. In the hypoxic TME, CAFs, tumour cells, endothelial cells and immune cells actively interact with each other. This complex interaction intensify the complexity of their paracrine signalling responses. CAFs secretes various cytokines, chemokines and pro-angiogenic factors in settled tumours, containing inter alia IL-10, IL-8, IL-6, tumour necrosis factor (TNF), TGF-β, C-C motif chemokine ligand-2 (CCL2), CCL5, CXCL9, CXCL10, SDF1, HGF, prostaglandin E2 (PGE2), nitric oxide (NO), HGF and human leukocyte antigen-G (HLAG); these secretions may have direct or indirect effects on tumour immunity as shown in *figure 1.9* [75].



Figures 1.9: *CAFs immunomodulatory function in tumour. CAFs secrets various chemokines, cytokines and growth factors to reshape the TME and regulate both innate and adaptive immune cells and transform them to pro-tumour phenotype. Ziani et al,2018).Adapted Frontiers in immunology 9, 414 (2018)[48].*

CAFs-derived IL-6 involved in the differentiation of monocyte towards macrophage instead of dendritic cells [76] and that CAFs-secreted cytokine also involve in the enlistment and activation of mast cells [42]. One of the *in-vitro* based studies showed that CAFs derived IL-4, IL-6 and IL-8 may stimulate the immunosuppressive myeloid cell differentiation [77]. CAFs secreted CXCL14 involve in the macrophage recruitment towards a tumour [78]. All these studies mentioned the CAFs-immune cell interact with each other via paracrine signalling, which is very crucial for the proliferation and development of a tumour [79]. CAFs secreted CXCL9, CXCL10 and SDF1 involve in the recruitment of T-cell [78]. CAFs-directed TGF-β can also modulate the function of T-cells by hindering the key genes involved in their cytotoxic activity (perforin, granzyme A and B, FAs ligand and IFN-γ)[80, 81] as well as cause the death of cytotoxic T-lymphocyte by suppressing the pro-survival protein Bcl-2 [82]. CAFs can also create an immunosuppressive TME via

generation of its metabolic reprogramming factors (IDO1, galectin, Arginase-2), that cause T-cell anergy and apoptosis [82-84]. Meanwhile, CAFs-directed TGF-β decreases the production of IFN-γ by NK-cells, which is critical for priming the effector CD4+-TH1 cells to kill tumour cells [85, 86]. Various investigations about melanoma, colorectal and hepatocellular carcinoma have demonstrated that CAFs-secreted PGE2/IDO can regulate the manifestation of NK-cell activating receptors, (NKp30, Nkp44 and NKGD2) as well as perforin and granzyme-B expression by NK cells, that can suppress the NK-cell cytotoxic action against tumour cells [87, 88]. In one of the subgroups of CAFs from lung cancer patietnts exhibited PDL-1 and PDL-2 on their surface and may involve in the T-cell suppression *ex vivo* [89]. CAFs also regulate the tumour immunity indirectly via its effect on tumour-angiogenesis [90]. CAFs and tumour cells also limit the activity of immune cells towards via constructing ECM-network. These ECM remodelling releases various pro-inflammatory cytokine, growth factors that can assist in immune cells collection [91]. Along these lines, CAFs can regulate the immune cells around tumour cells to create an immunosuppressive TME. In the present study, we are also trying to investigate the immunoregulatory function of CAFs on monocyte-derived macrophages.

1.3 Macrophages

Macrophages are a functionally and phenotypically prominent populations of innate immune cells, which are crucial for tissue development and homeostasis [92]. They are available around every tissue and body organ that portrays a higher degree of heterogeneity and plasticity. Some populations of macrophages exist in numerous tissues, such as brain, lungs and liver. They are originated from the yolk sac or foetal liver during early development of the organism. In general, macrophages are recognized as bone marrow-derived cells of myeloid lineage that circulate in the blood as monocytes. In acute inflammatory condition, monocytes leave the bone marrow and circulate in the blood for one to two days. After that, these circulating monocytes enter the peripheral tissues, where they became transformed into macrophages[93].

Moreover, macrophages are an integral part of innate immunity, and they perform various functions. As a professional phagocyte, they are involved in the phagocytosis of microorganism and efferocytosis (phagocytosis of apoptotic cells). In this way, they constantly remove senescent cells, apoptotic cells and cellular debris from the body. Macrophages are considered to be one of the major secretory cells in the body. The various cytokine, chemokine, and growth factors they produce influences tissue development and angiogenesis. Furthermore, they affect the remodelling of extracellular membrane content by releasing several metalloproteinases. Activated macrophages

are the primary source of toxic intermediates, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), involved in the antibacterial and cytotoxic functions. Their role as antigen-presenting cells involves displaying the antigen to T-helper cells and acting as a bridge between innate and adaptive immunity [94].

1.3.1 Macrophage Polarization

Macrophages are versatile cells, with the capacity to change their functional status according to the environment. They may perform immune effector roles in host defence and at the same time a homeostatic role to maintain the balance. This dual role of macrophages has been explained through a model (as portrayed in *Figure1.10*), which describes the macrophage activation as a polarisation of two states: the M1 or classical, and the M2 or alternative activation [93].

Figure1.10: *Linear classification of macrophages: Classically activated macrophages (M1) are at one end and alternatively activated macrophages (M2) at the other end. The gray area in between explians the wide of phenotypes that macrophages can adopt. Derived and modifies from Mosser,D.M and J.P.Edwards (2008)[95].*

In the background of the varying macrophages activation stages, a wide array of transcription factors, epigenetic factor and post-transcriptional regulators, are involved [96, 97]. In the perspective of the latest research and new development in immunology, some researchers argued that this polarisation of macrophages is oversimplified and there is a need to reassess this classification [93].

1.3.1.1 Classically Activated Macrophages (M1)

The classically activated macrophages are the set of macrophages that is activated with pathogen-associated molecular pattern molecules such as LPS, or endogenous/exogenous danger signals [98] or interferon –gamma (IFN-γ), to differentiate into an M1 phenotype. Interferon gamma is the primary signal that primes the macrophage towards the M1 phenotype [99]. LPS act through Toll-like receptor (TLRs) 2 and 4 [94], which are manifested in high amounts in M1 macrophages.

Moreover, the M1 macrophage is the well-illustrated phenotype which can initiate the T-helper cell polarisation, into TH1 response. TH1 response is described by the generation of interferon–gamma (IFN-γ), tumour necrosis factor (TNF- α), and Interleukin-2 (IL-2), which trigger the bactericidal activity of macrophage and prompt B-cell to make opsins and complement-fixing antibodies [94]. They act as professional phagocytic and antigen-presenting cells in the body. Metabolically in these macrophages, inducible nitric oxide (iNO) catalyzes the conversion of L-arginine to NO and reactive oxygen species and is used as M1 marker [100]. In addition to the secretion of Th1 pro-inflammatory cytokines including IL-1, IL-6, IL-12, IL-23 and TNF-α. These macrophages have a strong cytotoxic capacity and anti-proliferative impact through the production of reactive oxygen species (ROS) and nitric oxide (NO) [96].

1.3.1.2 Alternatively- activated macrophages (M2)

On the opposite, macrophages may be activated through TH2 cytokines (IL-4 and IL-13) [96] named as M2 macrophages. Several other factors like dexamethasone, IL-10, and transforming growth factor beta (TGF-β) also induce the M2 polarisation. Given their diversity, M2 macrophages are further classified into other categories based on the nature of their stimulants- M2a, M2b, M2c and M2d as illustrated in *Figure 1.11*.

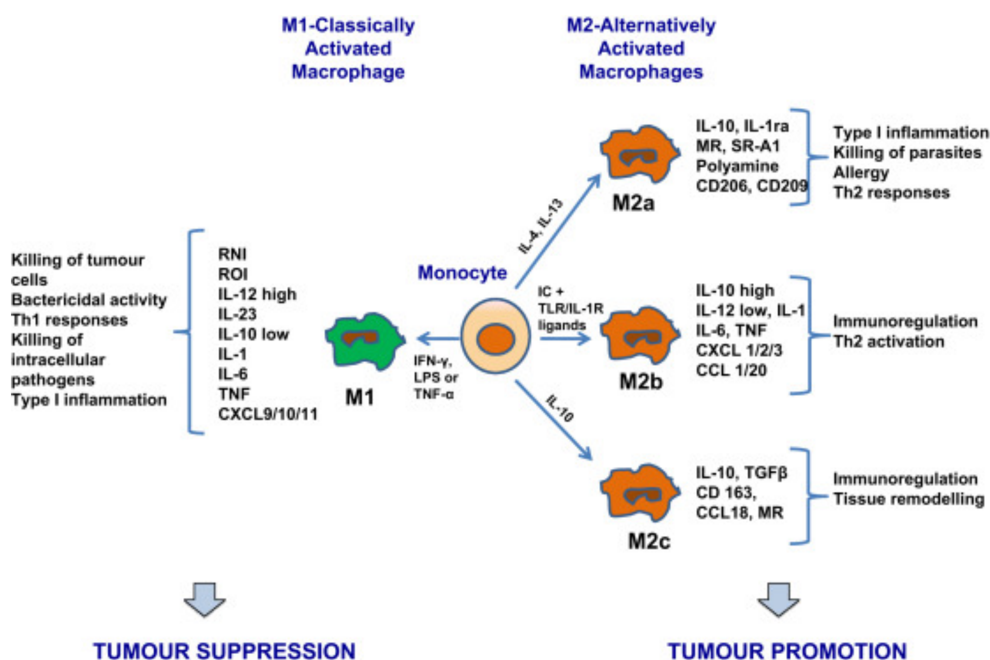


Figure 1.11: *The polarization of monocyte into M1 and M2 macrophages and their function is described in this picture. Retrieved and modified from Vasiliadou et al 2013 (122)[101].*

M2a phenotype is induced primarily through IL-4 or IL-13 stimulation. M2b phenotype is induced by immune complexes and Toll-like receptor ligands, or IL-1receptor antagonist (IL-1Ra). M2c

phenotype is triggered by IL-10, TGF-β1 or glucocorticoids, while M2d phenotype is initiated through Toll-like receptor and adenosine A2A agonist.

The M2 macrophages are significant regulators of inflammation and wound healing [102]. They produce several growth factors like TGF-β and Platelet-derived growth factor (PDGF). These factors stimulate the proliferation of epithelial cells and fibroblast [103]. M2 macrophages also influence the expression of matrix metalloproteinases (MMPs) and tissue inhibitor metallo-proteinases (TIMP) [103], which enhances the remodelling of tissues. They stimulate the angiogenesis through the release of a pro-angiogenic mediator like vascular endothelial growth factor (VEGFs) [104]. Moreover, M2 macrophages display a wide variety of scavenger receptors that mediate the endocytosis of macromolecules [105, 106]. Ligands of the scavenger receptor are present in the cell wall of gram-positive bacteria (nucleic acid, lipoteichoic acid, oxidised lipoprotein, sulphate polysaccharides and extracellular matrix components ECM) [107]. Metabolically, the M2 macrophage manifests high levels of arginase, which catalyses the conversion of arginine and water into ornithine and urea, it is therefore regarded as M2 marker.

Recent research propose that different stimuli may induce different phenotypes in macrophages, [108] such as haemorrhage-associated macrophages (Mhem) which are induced by haemoglobin [109]. Mox macrophage is generated with oxidised phospholipids, and M4 macrophage produced by chemokine ligand-4 [109]. There is another category named as regulatory macrophages, portrayed by a FoxP+ expression, that has been reported among tumour-associated macrophages [108]. These macrophages have been claimed to participate in homeostasis by constraining the inflammatory immune response and prolonging the classical macrophage activation [109].

It is also speculated that regulatory macrophages are similar to M2 macrophages by constraining tissue damage. They are, however, not involved in wound healing [109].

1.3.2 Macrophages role in tumour progression

TAMs are recruited into the tumour microenvironment under the influence of various chemokines (SDF-1, CCL-2) and growth factors (M-CSF, VEGF). Traditionally, macrophages were regarded as anti-tumorigenic (M1), repressing tumour growth, but TAMs show more frequently pro-tumorigenic characteristics as portrayed in *Figure 1.13*.

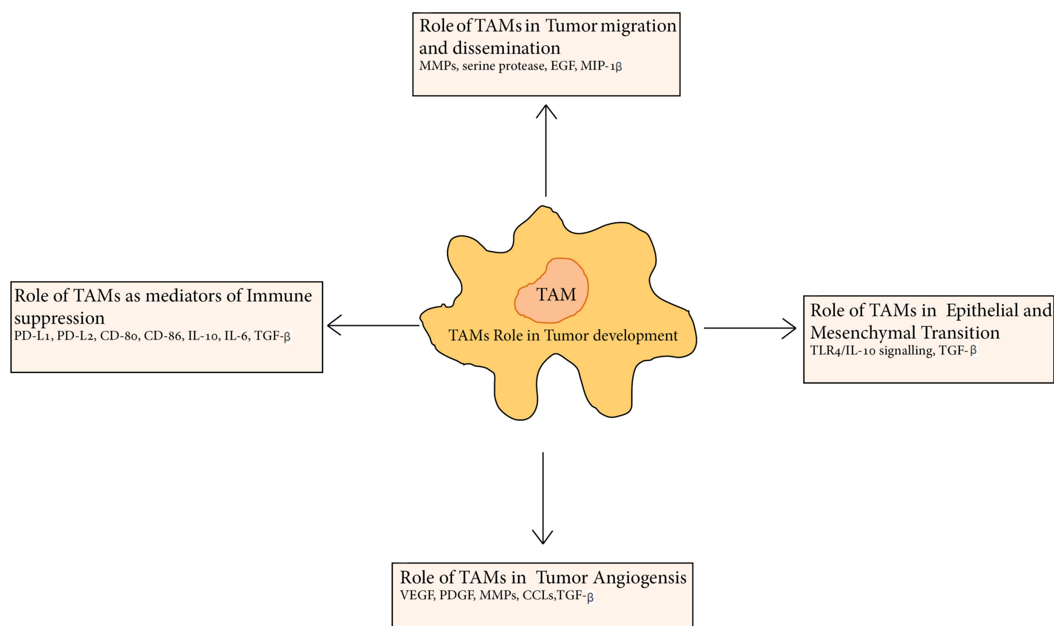


Figure 1.13: *Role of TAMs in Tumor Development in tumor proliferalrtion and metastasis are described here. Retrieved and modified from Aras et el 2017 [110].*

TAMs are considered pivotal controllers of all phases of metastasis, and help the tumour cell in local invasions, intravasation into blood vessels, and extravasation to the peripheral tissue via various secretions such as matrix metalloproteinases, serine proteases, and cathepsins [111]. These secretions adjust the composition of the extracellular membrane by changing the cell-cell junction and advancing the basal membrane disruption, which assists in local invasion and tumour escape [112]. Mechanistically, the secretion of CSF-1(colony stimulating factor-1) by tumour cells triggers the TAMs to produce EGF (epidermal growth factor), which in turn facilitate the tumour cell migration and intravasation [113].

A current report manifested that, Warburg effect in TAMs propelled vascularisation, extravasation, and metastasis in the patient of adenocarcinoma. Blocking of glycolysis in TAMs with a suitable inhibitor disturbed this metastatic phenotype and turning off this observation of an increase in TAMs assisted angiogenesis, extravasation and epithelial-mesenchymal transition (EMT) [114].

EMT assumes a fundamental part in tumour expansion and metastasis where polarised epithelial cells alter their appearance to mobile mesenchymal cells. The latest investigations demonstrate that

TAMs are important players of this process, which assists in the deprivation of cell to cell adherence and the acquisition of a mobile phenotype through the release of growth factors and cytokines (TGF-β, HGF, EGF, Notch, Wnt and NFkB) [115]. One of the studies explained that M2-phenotype TAMs advanced the EMT in pancreatic cancer, partly via TLR4/IL-10 signalling pathway [110].

On the other hand, mesenchymal-like cancerous cells released granulocyte-macrophage colony stimulating factor (GM-CSF), which can polarise the surrounding macrophage into TAMs. TGF-β secreted by the TAMs [116] or tumour cell under the stimulation of macrophage-derived cytokine (IL-6, IL-1 β and TNF-α) is another significant inducer of EMT in malignancy [117]. Multiple transcription factors are involved in TGF-β induced EMT such as Snail, ZEB, and bHLH families. TAMs are also the vital source of Matrix metalloproteinases, especially MMP-9. MMP-9 contributes in EMT by disrupting tissue structure and creating space for a developing tumour. MMP9 can also liberate IGFs (Insulin-like growth factor), prominently IGF-1, which give survival signal to the cell, including cancer cells [118].

Furthermore, TAMs also stimulate the EphA4 through nuclear translocation of NF-κB. This is known to prompt the production of an active cytokines such as IL-16, IL-18 and GM-CSF, and along these lines keeps the stem cell-like attributes of cancerous cells and assist in EMT [119].

The progression of a tumour from benign to a malignant state depends on the expansion of vascularisation, known as **angiogenesis**, which supplies nutrient and oxygen to the cancer cells and enables them to proliferate, and metastasise. Due to its importance, angiogenesis is considered a hallmark in cancer [119]. TAMs released pro-angiogenic factor like VEGF, PDGF, TGF-β, and matrix metalloproteinases (MMPs) assist in neovascularization of tumours. It was documented that VEGF-A is involved in neo-angiogenesis and macrophage enlistment to the tumour site [120]. It propels the development of irregular vasculature in tumours, creating many branches with dead ends and leakiness that affect a tumour haemodynamic and drug distribution[121, 122]. The specific removal of VEGF-A in macrophages using lysozyme uncovered their contribution in the abnormal vasculature in tumours [123].

TAMs are engaged in immunosuppressive activity either by producing immunosuppressive factors such as prostaglandin, arginase-1, IL-10 and TGF-β. For example, TGF-β is a multipoint growth factor which advances the shift of M1 to M2 phenotype in TAMs. This, in turn, inhibits the

function of NK cells, the migration of dendritic cells and their antigen presenting capability [124, 125]. Moreover, TGF-β, stimulates the helper T-cells (CD4+) towards the Th-2 response rather than Th-1 response and inhibits the cytotoxic T-cell (CD8+) activity [81].

Also, IL-10 (which is secreted by TAMs, cytotoxic T-lymphocyte and tumour cell) is an imperative mediator in the tumour microenvironment and plays an immunosuppressive and anti-inflammatory role that favour the escape of a tumour from immune surveillance. IL-10 secretion by TAMs acts in an autocrine lope and represses the secretion of IL-12 [126]. Likewise, IL-10 also suppresses the expression of IFN-γ and dendritic cell maturation [127]. It also diminishes the antigen presenting ability of professional phagocytes about a tumour related antigen [128].

TAMs also express COX-2, which leads to the immunosuppression and tumour progression [129], as COX-2 (cyclo-oxygenase 2) is involved in the conversion of arachidonic acid into prostaglandin endoperoxide (prostaglandin E2). Macrophage also exhibits numerous ligands, PD-L1/PD-L2 and CD80/CD86, which connect with lymphocyte inhibitory receptors as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen (CTLA-4) respectively. The CAFs (cancer-associated fibroblast) was also induced the aggregation of TAM. The secretion of IL-10, TGF-β, and arginase in oral squamous cell carcinoma advances the immunosuppressive microenvironment by repressing the T-cell multiplication[130].

Tumour-associated macrophages also suppress the effector T-cells function by recruiting the regulatory T-cells in their vicinity via CCL22 [81]. In vitro studies, these T-regulatory cells instigate the IL-6 and IL-10 expression by macrophages, prompting the autocrine upregulation of B7-H4 and suppressive phenotype [127].

1.4 Impact of radiation in the tumour microenvironment

Radiotherapy is applied to treat more than 50% of diagnosed cancer patients and accounts for up to 40% of total cures [131]. For a long time, the target of radiotherapy has been focused solely on the tumour cell itself, neglecting the surrounding microenvironment in which a tumour grows. In the recent past, new perspective of radiobiology emphasised the importance of TME. In the following chapter, I elaborate on how ionizing irradiation treatment affects the immune response of tumour cells and its related stroma.

1.4.1 Radiotherapy and tumor immunity

The ionizing radiation can destroy tumours cell directly by damaging DNA through the generation of free radicals. This DNA damage prompts the production and presentation of tumour-associated neoantigen [132, 133] in addition to the secretion of cytokines from a tumour and stromal cells [134]. In this way, it can expose the tumour cell to the immune system by immunomodulation of cell surface molecules. Radiotherapy has been shown to enhance the expression of MHC class I molecule [135, 136] and NKG2D ligands [136] on the tumour cell surface, which increase the chance of recognition by antigen-specific CD8+ T-lymphocyte and NK cell. Radiotherapy can also trigger the manifestation of FAS death receptor on tumour cells, increasing the ability of FASL (ligand) bearing T-cells to recognize and destroy the tumour cells [137].

All these proceedings can modify the immunosuppressive condition towards an immunoreactive microenvironment. Previously, it has been postulated that radiotherapy (RT) provide only immunosuppressive effects, because of the damage to tumour-infiltrating and the nearby immune cells, thus inducing lymphocytopenia [91, 138]. However, several documentations have shown that focal radiation may exert effects on distant tumour sites [139]. This radiation impact can be defined as an "**abscopal** effect" (off target). This abscopal impact can portray the radiotherapy as a pro-immunogenic effect, rather than immunosuppressive effect as displayed in *Figure 1.14*.

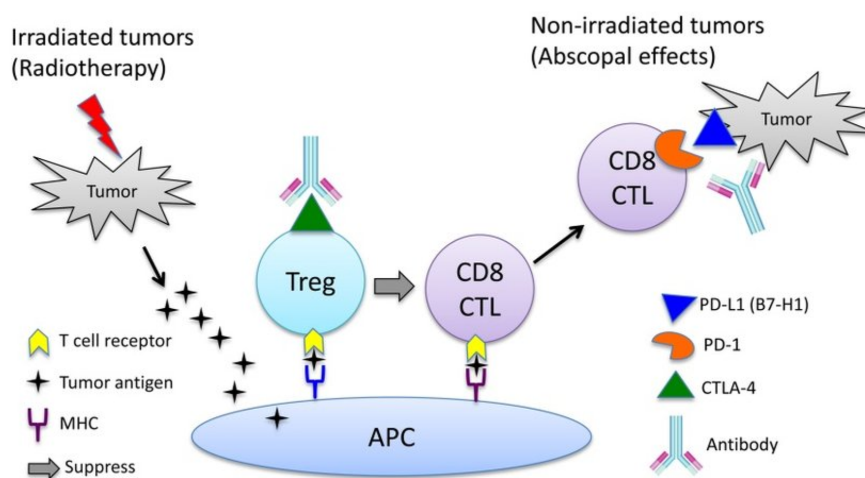


Figure 1.14: *Pro-immunogenic effect of radiation on tumour cells: Radiation therapy can induce the production of tumour antigen and make it visible to the immune system. After that, antigen presenting cells capture the tumour antigen and generate a specific immune response. This response is not limited to the primary tumour site, but it has an effect on distant tumour site (Abscopal effect). Adapted from Golden et al, 2012[140].*

Radiation can assist in cross-presentation of tumour antigens to T-lymphocyte through immunogenic cell death (ICD). ICD relates to the discharge of alarmins (danger signals) from tumour cells. Alarmins are endogenous danger signals that bind to Toll-like receptors (TLR) and prompt the dendritic cell maturation and amplify the MHC-class I and MHC-class II cross-presentation of tumour antigen to a tumour specific cytotoxic and helper T-lymphocyte appropriately. Following that, this activated T-lymphocyte come towards tumour mass and perform their effector function. Danger signals which interact with ICD incorporate the translocation of the protein, calreticulin (CRT), and liberation of high mobility group protein 1 (HMGB1), adenosine triphosphate (ATP), and heat shock proteins (HSPs) 70 and 90 related with stress cells into the extracellular milieu [140].

1.4.2 Effects of radiotherapy on TME components

1.4.2.1 Effects on the Vasculature:

Radiation leads to the destruction of endothelial cells, described by high permeability, separation from the basement membrane and apoptosis [141]. Apoptosis and the malfunctioning of vessels assist in fibrosis and post-irradiation inflammation. While inside vessels, irradiation induces a pro-thrombotic condition featured by the accumulation of platelets, microthrombus formation, and enhances the adherence of inflammatory cells to endothelial cells [142]. Morphological changes may be reversible or irreversible depending on the dose of radiation, size-type-stage and location of a tumour, which ultimately influences the efficacy of radiotherapy [143]. Radiation-induced vascular damages enhance tumour hypoxia and instigate immune reactions via high production of chemokine/cytokine that stimulate the enrollment of immune cells. Following a tumour, revascularisation happens through hypoxia-inducible factor-1α (HIF-1α)-dependant and independent enrollment of bone marrow-derived cells (BMDCs) [142, 143].

1.4.2.2 RT effects on the Stroma:

Chronic inflammation is tightly related to fibrosis, in that consistent immune responses appears beside tissue remodelling and repair procedure. In the normal tissue, invasion of inflammatory cells activates myofibroblast transformation, which results in the constant production of various growth factors, connective tissue growth factors, angiogenic/ fibrogenic cytokines, chemokines that finally lead to the over deposition of extracellular matrix (ECM) [144]. The inflammatory reaction that originates quickly after radiotherapy is not identical to the one described, but shares some common features, such as, activation of stromal cells, self-sustaining signalling networks [145]. Cancer with a prominent desmoplastic reaction showed naturally radio-resistant phenotype,

as a result of integrin-mediated connection among tumour cells, stromal cell and surrounding ECM [146].

Radiotherapy of a tumour also affects CAFs, and CAFs from non-small cell lung cancer (NSCLC) specimens lose their tumor-invasive potential after high dose RT [147]. Moreover, many investigations mentioned CAFs as a radio-resistant cell, which can sustain radiation doses above 50 Gy [148, 149]. Despite that, CAFs exposed to a single high dose of over 10 Gy of radiation develop a senescent phenotype due to irreversible DNA damage [121, 147]. These senescent fibroblast does not multiply, but they still produce various soluble factors to reshape the tumour microenvironment [150].

1.4.2.3 RT effects on inflammation and the immune system:

Radiotherapy always triggers an inflammatory response due to the instigated issue damage. However, after the initial proinflammatory phase, it starts the second phase in the context of tissue repair characterized by immunosuppressive activities, such as the recruitment of immunosuppresive cells and the release of immunosuppressive mediators [6]. After radiotherapy, the number of these immunosuppressive cells increase in the TME, as these cells are less radiosensitive than others lymphocytes [151]. These rise in the number of immune cells is counter-balanced by the recruitment of new circulating immune cells and increase in antigen expression [152].

Radiation can have a direct and indirect effect via direct enrollment of immune cells such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), selectin and activation of inflammatory cytokine cascade (IL-1, TNF-α)[153]. All these events mainly interconnected via reactive oxygen species (ROS) and NF-κB , which cause cellular stress and death[153]. These events lead to the induction of an immune response via the production of the damage-associated molecular pattern (DAMPs) and their corresponding pattern recognition receptors (PPRs)[153].

In a nutshell, the complicated response of immune system to an irradiated TME is not fully immune-stimulatory or immune-suppressive. It depends on the balanced production of inflammatory cytokines, dendritic cell priming and antigen presentation, along with the expansion of the radioresistant population of macrophages and T-lymphocytes. The complex signalling from both tumour ICD and radiation-induced-modification to endothelial cells give rise to the invasion of immune cells which are more sensitive to immune-stimulation. Hence, radiotherapy is not an

ideal procedure for stimulating ICD of tumour cells followed by effective adaptive reaction. Nevertheless, some important events, like the modification of immunoregulatory cytokines, dendric cell maturation, T-cell recruitment and activation, can have a potential to stimulate the effective immune response against a tumour, if the immunosuppressive response can be suppressed.

2 Aim of the study

It has been extensively demonstrated that cancer-associated fibroblasts can act as a powerful immunoregulatory cell, with the ability to transform the anti-tumour features of immune cells towards pro-tumour features. Moreover, in the context of radiotherapy, some studies reveal that CAFs exposed to high dose ionizing radiation can experience a broad and permanent phenotypic modification. While, CAFs exposed to a lower dose fractioned radiotherapy, induce reversible DNA damage without growth arrest [147].

On this background, in this thesis, we investigate the immunomodulatory features of primary CAFs on monocyte-derived macrophages *in vitro,* and explore if ionizing radiation, given at different regimens, is able to modify CAF-mediated immunoregulatory functions.

The experimental settings comprise the use of both, co-culture of the two cell types and incubations with CAF conditioned media. Analyses of macrophage phenotype and functions include expression of cell surface co-stimulatory receptors, production of nitric oxide and secretion of inflammatory cytokines.

3 Materials

3.1 Cell Culture Medium and supplements

Classical Media	Catalog number	Supplier	Origin
Dulbecco's Modified Eagle Medium (DMEM)	D6046	Sigma-Aldrich	USA
DMEM/F-12 (1:1)	31330-038	Gibco	UK
Iscove's Modified Dulbecco's Medium (IMDM)	BE12-722F	Lonza	Belgium
Broncho-epithelial Growth Medium Kit	CC-3170	Lonza	Belgium
Supplements			
Fetal Bovine Serum (FBS)	S0115	Biochrome	Germany
Penicillin-Streptomycin	P0781	Sigma-Aldrich	USA
Ascorbic acid	CAS:50-81-7	VWR Chemicals	Belgium

3.2 Reagents for cell isolation and culture

Bacterial-Collagenase	C9407	Sigma-Aldrich	USA
Enzyme-Free Cell Dissociation soln.	S-014-B	Millipore	USA
Trypsin-EDTA	T4049	Sigma-Aldrich	USA
Cell Freezing			

Dimethyl Sulfoxide (DMSO)	WAK-DMSO-10	GMBH	Germany
PBMC Isolation			
Lymphoprep™	01-63-12-001-A	Axis Shield	Norway
Cell Washing			
Dulbecco's Phosphate Buffered Saline (PBS)	RNBF9311	Sigma-Aldrich	USA
Bovine Serum Albumin	4J013790	AppliChem Panreac	Germany

3.3 Supplies

Plastic ware	**Catalogue number**	**Supplier**	**Origin**
96-Well plate white sterile plates	655073	Greiner Bio-One	Germany
Costar® Low attachment 24-well plates	CLS3473	Sigma-Aldrich	Netherlands
Falcon™Tissue culture 24-well plates	353047	BD Falcon	USA
Falcon™Tissue culture 6-well plates	353046	BD Falcon	USA
NuncPetriDishes	249964	ThermoScientific	USA
NuncEasyFlask 25cm²	156367	ThermoScientific	Denmark
NuncEasyFlask 75cm²	156499	ThermoScientific	Denmark

NuncEasyFlask 175cm²	159910	ThermoScientific	Denmark
Transwell cell culture inserts	CLS3464	Sigma-Aldrich	USA
Cell Freezing			
Nunc CryoTube Vials	363401	ThermoScientific	Denmark
Other Supplies			
Centrifugal Concentrator (VIVASPIN 6)	Vs0612	Startorius Stedim	Germany
Surgical blade	P308	PARAGON	UK
Syringe	300613	BD plastipal™	Spain
Syringe Filter Unit	SLHA033SB	EMDMillipore	Ireland
Nunc® inoculating loops	**I7773**	Sigma-Aldrich	USA

3.4 Stains and dyes

Name	**Catalog number**	**Supplier**	**Origin**
DAF-2DA	251505-1MG	EMD Millipore	USA

3.5 Antibodies for flow cytometry

mAntibodies	**Conjugate**	**Catalog Number**	**Supplier**	**Dilution**	**Origin**

Mouse anti-human CD80	PE	130-110-270	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD45	APC	130-110-635	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD86	FITC	130-116-159	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD40	PE	130-110-946	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD163	FITC	130-112-132	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD14	FITC	130-110-521	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human HLA-DR	PEVIOR615	130-111-950	MACS-Miltenyi Biotec	1:50	Germany
Mouse anti-human CD206	PE	555954	BD Biosciences	1:10	Germany
Mouse-IgG1	FITC	130-098-845	MACS-Miltenyi Biotec	1:50	Germany
Mouse-IgG1	PE	130-098-847	MACS-Miltenyi Biotec	1:50	Germany

3.6 Recombinant Proteins

Cytokines	**Catalog Number**	**Supplier**	**Reconstitution**	**Origin**
Recombinant Human IFN-γ	300-02	PeProTech	1 mg/mL in dH_2O with 0.1 % BSA	USA
Recombinant Human IL-4	500-M04	PeProTech	1 mg/mL in dH_2O with 0.1 % BSA	USA
Recombinant Human IL-10	200-10	PeProTech	1 mg/mL in dH_2O with 0.1 % BSA	USA
Recombinant Human M-CSF	300-25-50UG	PeProTech	50 µg /mL in dH_2O with 0.1 % BSA	USA
Recombinant Human TGF-Beta	100-21	PeProTech		UK

3.7 Kits

ELISAs Kits	**Catalog Number**	**Supplier**	**Origin**
Human IL- 12 DuoSet® ELISA	DY1240-05	R&D Systems	USA
Human IL-6 DuoSet® ELISA	DY240-05	R&D Systems	USA
Human TNF-α DuoSet® ELISA	DY210-05	R&D Systems	USA

Human IL- 10 DuoSet® ELISA	DY217B-05	R&D Systems	USA

3.8 ELISAs Reagents

DuoSet® ELISA Ancillary Products	**Catalog Number**	**Supplier**	**Origin**
Reagent Diluent Concentrate 1	DY997	R&D Systems	USA
Color Reagent A and B	DY999	R&D Systems	USA
Tween 20	P416	Sigma-Aldrich	USA
Sulphuric Acid (H_2SO_4) 95-95%	100731	Millipore	Germany
HEPES	H3784	Sigma-Aldrich	USA
Sodium Hydroxide (NaOH) (FW = 40)	71690	Sigma-Aldrich	USA
Wash Buffer (25x)	WA126	R&D Systems	USA
Reagent Diluent Concentrate 2	DY995	Sigma-Aldrich	USA
Normal Goat Serum	DY005	R&D Systems	USA
Tris (FW = 121.11)	72H5601	Sigma-Aldrich	USA
Sodium Chloride (FW = 58.44)	K26478104917	MERCK	Germany

3.9 FACS flow products

Reagents	**Catalog number**	**Supplier**	**Origin**

FACSFlow	342003	BD Bioscience	Netherlands
FACSRinse	340346	BD Bioscience	Netherlands
Tubes			
Falcon™Round-Bottom Polystyrene Tubes	352235	BD Bioscience	USA

3.10 MACS Cell Separation Products

Accessories	**Catalog Number**	**Supplier**	**Origin**
MACS MultiStand	130-042-303	Miltenyl Biotec	USA
Separator			
MidiMACS Separator	130-042-302	Miltenyl Biotec	USA
Reagents			
CD14 Microbeads	130-045-101	Miltenyl Biotec	USA
Columns			
LS Columns	130-042-401	Miltenyl Biotec	USA

3.11 Instruments

Bioluminescent image analyser	**Supplier**	**Origin**
Image Quant LAS 4000	GE HealthCare	Germany
Centrifuges		

Heraeus Sepatech Biofuge 13	Heraeus Sepatech GmbH	Germany
Multifuge X3R	ThermoScientific	Germany
Rotina 420R	Hettich	Germany
Centrifugal Vacuum Concentrator (Rotavapor)	Eppendorf /lifescience	Germany
Flow Cytometry		
FACSAria™	BD Bioscience	USA
FACSCalibur™	BD Bioscience	USA
Incubators		
37ºC Incubator	Termaks AS	Norway
HERAcell 150i (CO_2 cell culture incubator)	ThermoScientific	Germany
HERAcell 240i (CO_2 cell culture incubator)	ThermoScientific	Germany
Microplate Readers		
Bio-Plex® 200 systems	BIO-RAD	USA
EMax®	Molecular Devices	USA
Luminometer	Labsystems Luminoskan	USA
Multiskan Ascent	ThermoElectronCorporation	USA
Microplate Washer		
Well Wash 4 MK 2	ThermoElectronCorporation	USA

Filtration Vacuum manifold for bead washing	Pall® Life Sciences	USA
Microscope		
Inverted Microscope ECLIPSE TS100-F	NIKON	Japan

3.12 Software

Flow Cytometer	**Supplier**	**Origin**
CellQuest™Pro Software	BD Bioscience	USA
Flow Jo Office V7/8	Tree star	USA
FACSDiva Software	BD Bioscience	USA
Microplate Readers		
Ascent Software®	ThermoElectronCorporation	USA
Bio-Plex Manager™ 6.0 Software	BIO-RAD	USA
SoftMax® Pro Software	Molecular Devices	USA
Statistics Software		
Excel® 2018	Microsoft	USA
GraphPad Prism 7	GraphPad	USA
Image Soft Ware		
Spot Software 4.7	Diagnostic Instruments Inc.	USA

4 Methods:

Methods of the current study are explained in the following section. The flowchart presented in *Figure 4.1* gives a general view of the method used and followed in our study.

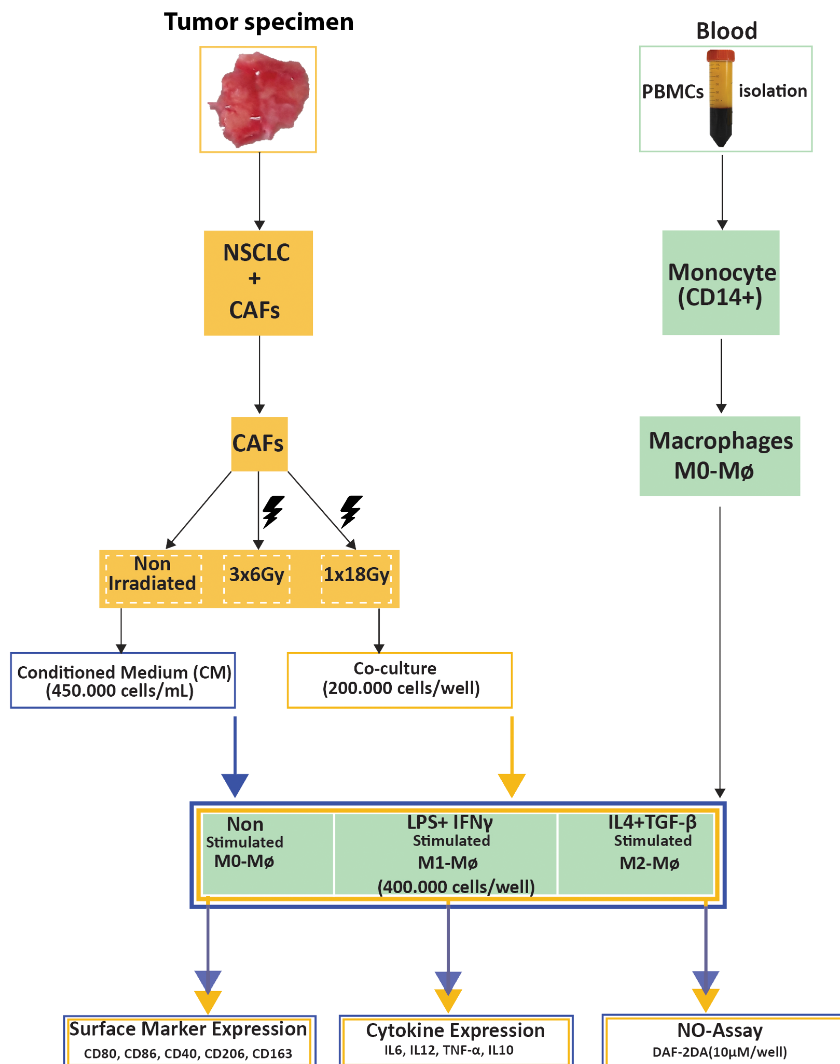


Figure 4.1: *Flowchart of pathways followed in the Current study: Experimentation started by collecting tumour specimen and blood buffy coat from the University Hospital Northern Norway. Followed by the isolation, culture and irradiation of primary CAFs from non-small cell lung carcinoma (NSCLC). Monocytes (CD14+ cells) were isolated from human Peripheral Blood Mononuclear Cells (PBMCs) and differentiated into macrophages. Macrophages were incubated with irradiated and non-irradiated CAFs-CM or co-cultured with irradiated and non-irradiated CAF-cells. Immunomodulatory effects of CAF-CM and CAFs in co-cultures on uncommitted M0 and pro-inflammatory M1 macrophages were observed through surface marker expression (flowcytometry), Cytokine secretion (ELISA), and Nitric oxide production (flowcytometry).*

4.1 Ethical Statement

The Regional Committee for Medical and Health Research Ethics (REK-Nord) approved the use of human material for this study (Project-ID: 2009/895-4), and informed written consent was obtained from all patients. All methods involving human material were performed in accordance with relevant guidelines and regulations.

4.2 Biological samples and Patients

Human lung tumour samples were collected from eight different patients diagnosed with NSCLC (Non-small cell lung carcinoma) at various stages and operated at the University Hospital of Northern Norway, Tromsø. Patients were not subjected to any additional therapy before sample collection. Furthermore, blood sample were collected from healthy blood donors attending the University Hospital of Northern-Norway Blood Bank. Information on patient demographics as well as tumor stage, size and histologic subtype is summarized in *table 4.1*.

Table 4.1 Feature of the CAFs donor used for the current study

Number	Age	Sex	Tumor Type	T-size (mm)	T-stage	N-stage
Donor 1	76	M	Adenocarcinoma	60 mm	3	1
Donor 2	71	M	Squamous Cell carcinoma	35 mm	2a	0
Donor 3	74	F	Adenocarcinoma	35 mm	2a	0
Donor 4	68	M	Squamous Cell carcinoma	22 mm	1c	0
Donor 5	71	F	Adenocarcinoma	25 mm	1	0
Donor 6	73	M	Adenocarcinoma	26 mm	1c	0
Donor 7	65	M	Squamous Cell carcinoma	30 mm	3	0
Donor 8	78	F	Squamous Cell carcinoma	18 mm	1b	0

Abbreviations: *F: Female; M: Male N0: There is no spread to nearby lymph nodes; N1: The cancer has spread to lymph nodes within the lung and/or around the area.*

4.3 Isolation and Culture of Primary NSCLC Fibroblasts

General Procedure:

Tumour specimens were collected from operated lung cancer patients (NSCLS).. Tumour tissue was collected in tubes containing ice-cold DMEM and immediately transported to the lab for further processing.

A. Tissue digestion

Tumour tissue samples were chopped into tiny pieces (1-1.5 mm3) in a petri dish with the use of surgical blades. These small pieces were collected into a T-25 cell culture flask and subjected to enzymatic digestion for 90 minutes with 0.8 mg/ml of bacterial collagenase in a final volume of 10 ml DMEM/F-12 by putting the T-25 flask on a shaker in a standard incubator at 37 ºC. A continuous shaking was provided to the digested solution to liberate all possible cells, trapped in the small piece of tissues. After that, cells and undigested material were collected in a 50 mL of sterile tube and spun down at 350 x g for 7 minutes to remove the collagenase. Supernatant was discarded, and the pellet was resuspended in 24 ml of fresh growing medium containing DMEM supplemented with 1% penicillin streptomycin and 10 % Foetal Bovine Serum (FBS).These cell suspensions were poured into 6-well small tissue culture plate (2ml/well) and placed into the cell culture incubator at 37ºC with low oxygen (3 % O_2) overnight, allowing all living and functional cells attach to the solid substrate.

B. Cell purification and culture expansion

After 2-3 weeks of incubation, the culture was a mix of fibroblasts and tumour cells. In order to obtain pure fibroblasts cultures, wells were washed once with pre-warmed PBS followed by 10 min incubation in 2 mL of Enzyme-Free cell detachment solution at 37ºC. This step promotes the selective detachment of fibroblasts from the plate. Then, the six-well plates were observed under the microscope to make sure fibroblast detachment. In case of poor detachment 500 µL of pre-warmed Trypsin-EDTA (0.25 % - 1mM) solution was added to the wells for 1 minute.

Enzyme-free solution detach the cells by keeping up the structural integrity of the cell surface protein, while Trypsin-EDTA detach the focal adhesion that are anchoring the cell to the flask. Detached cells were then collected in a 15 mL of tube, spun down at 350 x g for 7 minutes and resuspended in 10 ml of growth medium (DMEM +10% FBS). The cell suspension was divided into two T-75 cultural flask and 5mL of more growth medium were added into each flask. After

that flasks were placed for further propagation in a cell culture incubator at 37ºC in a 5 % CO2 humidified atmosphere.

C. Cell passage

Fibroblasts culture were observed under the microscope after every 24 hours and the growth medium was changed with fresh medium every 2-3 days until they reached 80-90% confluency. Fibroblasts have elongated shape and are anchorage dependent, which means that they grow in a monolayer while attached to a solid or semisolid substrate.

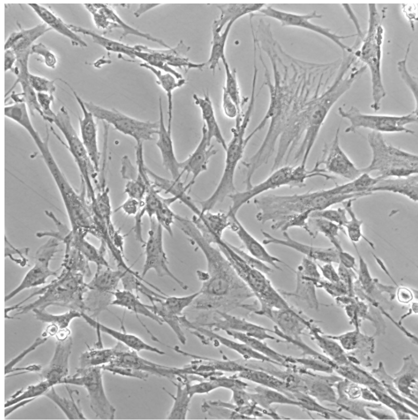


Figure 4.2 *Cancer Associated Fibroblast (CAFs) in culture in passage 1: cytoplasmic extension.* ***Image was captured with an inverted microscope connected to SPOT 4.7 image software.***

After attaining confluence, the medium was discarded, and the cells were detached using 5mL enzyme-free cell detachment solution for 5 minutes at 3% O_2. The cell aggregation was prevented by adding 1 mL of Trypsin-EDTA (0.25 % - 1mM) solution in the last minute. After that cell solution was collected in a 15 mL tube, spun down, resuspended with 10 mL of growth medium, divided in two T-175 flasks with 15 mL final volume of complete DMEM medium and incubated at 37ºC in a 5 % CO_2 humidified atmosphere.

D. Cryopreservation of primary tumour fibroblasts

After attaining the desired confluence (two passage), the cells were directly used for analysis or cryopreserved and used in later experiments. For cryopreservation, the cells were detached with enzyme free solution and Trypsin-EDTA (0.25 % - 1mM) solution as previously described, counted in a counting chamber, spun down and cryopreserved at 80ºC in freezing medium consisting of DMEM with 10% Dimethyl sulfoxide (DMSO) and 20 % FBS at 5x10^5 cells per mL per Cryotube.

DMSO is one of the common cryo-protectant used in the laboratory to prevent the formation of ice crystals during the freezing process which can damage the cell membrane. But DMSO can be toxic for the cells at high concentration (> 10 %) or if the cells are exposed to DMSO for a longer

period. That's why, to get the best survival rate, the thawing of the cryopreserved cells was carried out as quickly as possible. Once the CryoTube was removed from the freezer, it was placed into a 37ºC water bath until it was completely thawed. The cell suspension was then slowly added (drop by drop) in 10 mL pre-warmed complete medium. Cells were then seeded in a T-75 flask at 37ºC, in a 5 % CO_2 humidified atmosphere and incubated for 24 hours for attachment, the next day the medium was replaced with fresh medium to completely remove the DMSO.

4.4 Irradiation and preparation of Fibroblasts-Conditioned Medium

Conditioned medium was collected from irradiated and non-irradiated cultured CAFs in order to examine fibroblast paracrine effects on macrophages. Both irradiated and non-irradiated cell cultures were processed in an identical conditions.

General Procedure:

During the 3rd passage, CAFs were seeded at 4 x 10^5 cells per T-75 flask and incubated for 24 hours for attachment to the surface at 37°C in a 5 % CO2 humidified atmosphere. After the initial cell attachment and desired confluence (60-70%), (ca. 2-3 days after seeding), the medium was removed and 6 mL of complete DMEM medium with 10 % FBS was added followed by irradiation. CAFs were irradiated with high - energy photons beam of 15 megavoltage produced by a Varian clinical linear-accelerator derived as a single dose of 18 Gy (1 x 18 Gy) or triple dose of 6 Gy (3 x 6 Gy).

Figure 4.3 Varian clinical linear-accelerator. *Image is showing the 24 well plates with cultured fibroblast. The gantry was positioned below the flask.*

In the fractionated regimen, there was a gap of 24 hours between subsequent radiations. For a beam produced by 15 MV the dose from photons to tissue is at its maximum at 30 mm depth.

Therefore T-75 flasks were placed in the centre of a 20 x 20 cm field size of 30 mm depth consisting of 3 water/tissue-equivalent Perspex-plates. Each tissue-equivalent plate was 10 mm depth (i.e., 30 mm depth). The Gantry, which transports the beam from the linear-accelerator to the target, was positioned below the field *(Figure 4.3).*

For the experimental groups that received a single radiation dose, fresh culture medium was replaced 3 days post-irradiation and collected 6 days post-irradiation in 15 mL tubes, centrifuge at 2.200 x g for 5 min and passage through a 0.22-micron filter for elimination of potential contaminant cell bodies. CM was either used immediately for experimental analysis or frozen at –80ºC for further analysis in Eppendorf Protein LoBind tubes (1 mL/tube). Collected supernatants were normalized against number of cells in flaks. The cell density from the CM was at ~ 450.000 cells/mL.Before analysis, CAF-CM was thawed and vortex for 30 seconds to ensure adequate mixing of the protein suspension. Supernatants from non-irradiated CAFs were also conditioned for 3-days at a density of ~ 450.000 cells/mL. Of importance, control cells were not allowed to reach over confluence before harvesting since cells could enter the senescence state.

4.5 Isolation and Culture of Human Macrophages from Peripheral blood mononuclear cells (PBMCs).

Bags of 50 mL containing fresh buffy coats from healthy donors were collected from the hospital, and PBMCs were isolated using a Lymphoprep™ (Axis-Shield) density gradient centrifugation method.

General principles:

Lymphoprep™ is a density gradient method through which cells were speared according to their density. The blood cells which have higher density than medium (1.077 g/mL) were sediment through the medium, for example red blood cells and polymorphonuclear cells, while the other cells such as mononuclear cells which have less density than this medium were retained in the interface by centrifugation.

4.5.1 PBMC isolation procedure

The blood buffy coat bag (50ml) were collected from UNN hospital which opened carefully with surgical blade and pour it into 75 cm2 culture flask. Blood was diluted 1:1 with 0.2% PBSA (bovine serum albumin in PBS) to make a final volume of 100 ml. 15 mL of Lymphoprep™ solution were poured in 4x50mL tubes. Following that, we carefully added 25 ml of blood (blood + PBS) on top of 15 mL of Lymphoprep™ e, through a proper care to avoid the mixing of blood and

Lymphoprep™ solution. After that, centrifugation of all tubes were carried out at 800 x g, RT, without breaks (Acceleration:1 deceleration :1) for 30 minutes. Following that, the PBMC interface was collected with a Pasteur pipette, and washed three times with 40 ml of PBSA by centrifugation to eliminate the platelets. After last washing step, supernatants were discarded, and all cells were filtered through pre-separation filter in one tube. After counting, PBMC were divided into two fractions. One fraction was used for the isolation of monocytes (CD14+) cells and rest of PBMC were freeze down in a freezing medium containing 90 % FBS and 10 % DMSO. The cell density was $4x10^7$ cell per cryotube (1ml/tube) and cryopreserved at – 80ºC.

4.5.2 Immune-magnetic Monocyte Isolation

Anti-CD14-coated microbeads were used for the positive selection of human monocytes from PBMCs by following the principle of magnetic activated cell sorting separation(MACS).

The principle of the MACS separation is to isolate specific cell populations by particular surface antigens (CD molecules). In this process, samples are incubated with magnetic microbeads coated with monoclonal antibodies against a particular CD molecule. Then, the cell solution is loaded onto a MACS column which is placed in a strong magnetic field. Labelled cells with the magnetic microbeads are retained within the column while the unlabelled cells flow through. When the column is removed from the magnetic field, retained cells are eluted as the positively selected cell population.

Procedure:

The fraction of PBMCs selected for monocyte isolation were centrifuged at 300 x g for 10 minutes and re-suspended into 80 µL of MACS buffer (containing 0.2 % bovine serum albumin) per 10^7 cells. After that, 20 µL CD14 microbeads were added, mixed well and incubated for 15 minutes in the refrigerator (4°C). Following incubation, cells were washed with 1-2 mL 0.2% PBSA buffer and centrifuge at 300 x g for 10 minutes. Supernatant were aspirated, and the cells again resuspended into 500 µL of buffer. After that, cells suspension was loaded onto a MACS-LS-column inside the strong magnetic field. Following that, samples were rinsed with 3mL of 0.2% MACS buffer (0.2 % bovine serum albumin) onto the column. Then, we removed the column from the separator, and added 5mL of MACS buffer over it. We collected the magnetically labelled cells by firmly pushing the plunger into the column. As a last step of monocyte isolation, the eluted cells were washed with cold MACS buffer and went for centrifugation at 300 x g for 10 minutes. The cell fraction was resuspended in the 10 mL of cell culture medium (RPMI-1640 culture

medium plus 10 % FBS plus 1 % penicillin-streptomycin) and no of monocytes were counted and analysed by flow cytometry. (Labelling the antibody against CD14-FITC).

4.5.3 Monocyte differentiation into Macrophages

For the differentiation of monocytes (CD14+cells) into macrophages, a concrete amount of monocytes (6x106 monocyte) were re-suspended into 10 mL of cell culture medium (RPMI-1640 culture medium plus 10 % FBS plus 1 % penicillin-streptomycin) and pour into medium sized petri dishes (83mm) at the concentration of 6x106 monocytes. The petri plates were put into the incubator for 30 minutes so that cells were attached to the surface of the petri plates. After that, macrophage colony stimulating factor mCSF (100 ng/mL) were added into the culture medium for the conversion of monocytes into macrophages called non-stimulated macrophages (MO-Mϕ phenotype). These monocytes were cultured for six days with a medium change after three days. At the sixth day, cells were harvested by using enzyme free solution (10 mL) and the small amount of Trypsin (500 mL). Cell scraper was used very gently to scrap the cells from petri plate. Than petri plate checked under the inverted light microscope to confirm the proper harvesting of macrophages (70% of the initial number of cultured monocytes) from the surface. It varied according to donor.

After macrophages harvesting and counting, these macrophages (MO-Mϕ phenotype) were polarized by different stimuli for 48 hours. LPS (100ng/mL) and IFN-γ (20 ng/mL) were used for stimulation the M0 macrophages into M1 macrophages. While for polarization into M2 phenotype, the cocktail of IL-4 (50ng/mL) and TGF-ß (10 ng/mL) were used for 48 hours.

4.6 Cell Surface markers expression by flow cytometry

Cell surface markers represents proteins exclusively expressed on the surface of the cells and regularly used as markers of specific cell types. For the characterization of each phenotype of macrophages, a panel of various surface markers was used. These surface markers were specific for the particular phenotype of macrophages. For the detection of M0, M1 and M2 phenotype six different cell surface marker were used. HLADR, CD-40, CD-80 and CD8-6 markers were used for the characterization of M1 phenotype. While CD-206 and CD-163 were used as a marker of M2 phenotype.

Procedure:

For the characterization of macrophage phenotype, both stimulated and non-stimulated macrophages were collected in a 15 mL tube and counted. After that, macrophages (400,000 cells)

were suspended into 500 μL of MACS buffer and the 10 μL of antibody was added to the suspension (according to manufacture protocol), mixed well and incubated for 15 minutes in the darkness at 4°C. After 15 minutes, the cells were washed by adding 1-2 mL of buffer and centrifuged at 300 × g for 10 minutes. The pelleted cells were resuspended into 300 μL of MACS buffer and analysed by flow cytometry. PI were used to measure the live and dead cell ratio into the experiment.

Flow cytometry:

Flow cytomtery is a procedure applied to identify, count and measure the physical and chemical features of a population of cells/particles. The fundamental principle of flow cytomtry is the passage of cells in a single unit in front of a laser, so they can be identified and counted. The desired cells populations are fluorescently marked, and after that excited by the laser beam that emitted light at different length range. The transmitted light in the forward direction from the passing cells are typically used to identify the cell size, while the side scattered light provide detail about granularity and complexity. We used this procedure to identify and analyse surface markers expression in macrophages (CD-80, CD8-6, CD-40, HLADR, CD-206 and CD-163).

The gating and cell identification methodology for our sample was as follows; cell doublets and clumps were eliminated by FSC-H vs. FSC-A. While FSC-A vs SSC-A were used to make gates and macrophage population were identified based on the expression of CD45+ cells. Data is analysed by a computer connected to the flow cytometry using a FACSDiva Software

4.7 Co-culture of CAFs with Macrophages

Co-cultured of Irradiated and non-irradiated CAFs from different donors with macrophages were carried out in order to study the immunoregulatory effects of CAFs on macrophages when the cells are in contact or close proximity.

Procedure:

Co-culture of CAF-cells into macrophages were divided into two part

- Isolation and culture of human macrophages
- Isolation, culture and irradiation of Cancer Associated Fibroblast

The procedure for the isolation of human macrophages and CAFs cell were described above.

After the step of monocyte differentiation into macrophages, and the preparation of irradiated and non-irradiated fibroblasts, cells were co-cultured in a 24 wells plate in triplicate manner. CAFs were used for co-culture experiments 24 hours after the last radiation cycle. Of importance, we also investigated the effect of low dose fractioned therapy (three multiple doses of 6-Gy radiation-

3x6Gy) vs. single high dose radiotherapy (one single dose 18-Gy radiation-1x18Gy) on the cell surface marker of macrophage.
In that experiment, four different CAFs donor were used for co-culturing with macrophages. In each experiment, 400,000 macrophages/well were co-cultured for 48 hours with 200,000 CAFs/well (both irradiated and non-irradiated) in a 1mL/well of culture media (RPMI-1640 culture medium plus 10 % FBS plus 1 % penicillin-streptomycin).

After 48 hours of co-culture of the macrophages and CAFs, the supernatant of the co-culture was collected from the each well. This supernatant was centrifuged at 4500 x g for 5 minutes and filtered from 0.2micron filter. This filtered supernatant was put into eppendorf tubes, marked well and keep it at -70°C, until analysis of the cytokine.

Following the collection of supernatants, co-cultured cells were harvested for flow cytometry analysis as follows. Enzyme free solution (300 µL) were added into each well of the 24-well plate and incubate for 10 minutes. After that, 100 µL of Trypsin was added and incubated for 2-3 minutes. After trypsin incubation, the cell scraper was used to scrap the cell gently. After cell scraping, the cells were collected into tubes and centrifuged at 300 x g for 10 minutes at 4°C. Cell pellets were dissolved in culture medium and stained for the different surface markers (Secction4.6). Labelled cells were analysed by flow cytometry, Fluorescent-Activated Cell Sorting (FACSCalibur™).

4.8 Incubation of Macrophages with CAF-CM

To investigate the immunosuppressive effect of CAFs mediated by paracrine signalling, we collected 72 hour conditioned media from -irradiated and non-irradiated cultured CAFs and then incubated with macrophages for 48 hours.

General Procedure:

In that assay, we incubated the monocyte differentiated macrophages (M0, M1 and M2 phenotype) in triplicate in 24 well plate with the irradiated and non-irradiated CAF-CM for the period of 48 hours. After the described time of incubation, supernatant and the macrophage cells were collected in the similar manner as described in the section 4.6. In that experiment, conditioned medium from five different CAF donors were used. In each experiment, 400,000 cells/well of Macrophage were incubated for 48 hours with 500 µL of CAF-CM of both irradiated and non-irradiated CAFs (450,000 cells/mL of CM).

The immunomodulatory effect of these irradiated and non-irradiated CAFs were studied through flow cytometry on the expression of macrophage surface markers (CD-80, CD-86, CD-40, CD-206 and CD-163), (section 4.6).

4.9 Nitric Oxide assay

Nitric oxide is a small, unstable molecule which is produced by specific enzyme called nitric oxide synthetases (NOS). This enzyme has three isoforms. Two of the isoforms can generate Nitric oxide at the site of inflammation. Endothelial nitric oxide synthetase (eNOS) is manifested in activated endothelial cells and generate NO, which can assist in vasorelaxation and vasodilation. Another isoform called Inducible nitric oxide synthetase, is mostly present in activated macrophages, wherein NO generation is critical for microbial cytotoxicity [154].

General Principal:

DAF-2DA (4,5-diamino-fluorescein diacetate) is a non-fluorescent cell membrane permeable compound; once inside the cell, DAF-2DA is deacetylated by esterases resulting in the production of 4,5-diamino-fluorescein (DAF-2), which is retained within the cell. The reaction product of DAF-2 with Nitric oxide results in a benzotriazole derivative, a fluorescent compound which can be analysed by flow cytometry.

General Procedure:

The production of nitric oxide was measured in two different experimental settings: co-cultures and incubation with CAF-CM.

Same procedure for harvesting the cells as mentioned earlier was adopted (section 4.6). After due timing (as described earlier), the cells were washed and resuspended in 500 µL of culture medium (RPMI1640 + 1 % penicillin-streptomycin, without appropriate FBS) containing 10 µM DAF-2 DA (Calbiochem) / well. The 24 well plate were incubated in an incubator at 37 ° C and 5% CO2 for 1 hour. Soon after incubation, the cells were detached (as describes above) and analysed on the FACSAria Flow cytometry.

4.10 Enzyme linked immunosorbent assays (ELISA)

General principles:

The sandwiched ELISA quantify antigenic protein between two layers of antibodies (i.e capture and detection antibody). The Capture antibodies of the ELISAs are attached to the flat surface of a 96well plate. And the streptavidin that detects and binds to detector antibodies is conjugated by an enzyme (Horseradish Peroxidase (HRP) which is detected by a substrate Tetramethylbenzidine

(TMB). The enzyme – substrate compound turns the substrate into a coloured product. ELISA was specific for the detection of one protein at a time.

General procedure:

Four different ELISAs were performed in this study to detect and measure the quantity of: IL-6, IL-12, TNF-α and IL-10.

The concentrations of IL-6, IL-12, TNF-α and IL-10 were determined from the supernatant of non-irradiated and irradiated CAF with macrophages (incubated for 48 hours).

The human TNF-α DuoSet® ELISA kit, human IL-6 DuoSet® ELISA kit, human IL-10 DuoSet® ELISA kit and human IL-12 DuoSet® ELISA kit was used in triplicates according to product protocol. But we need to repeat ELISA for some sample to adjust the dilution factor. Samples were diluted 1:10 in reagent diluent for the determination of IL-6. Samples were diluted 1:5 in reagent diluent for the determination of TNF-α, while no dilution was made for IL-10 and IL-12.

The reading of these plates was recorded through a microplate reader at wavelength of 450 nm. Ascent software were used for the recording of standard curve and for the estimation of unknown protein quantity in a sample. For curve fitting, again follow the product protocol.

All the reagents and solution that were used in that experiment were purchases individually and reconstituted according to the instructions. Two-fold serial dilution were used for establishing the standard curve and it was run in duplicates for all the standard reagents for all the ELISA test. The experimental control was containing of M0, M1, and M2 macrophage.

4.11 Statistical Analysis

All results are the average determination of multiple donors with standard deviations (S.D) and displayed as fold M0-control. In ELISA assays only the readings above the detection limit of the assays are illustrated in the figures. Data were analysed using paired student's t-test. The p value < 0.05 were statistically significant. All statistics were analysed with Graph pad prism 7.00 for MacBook Air. Graphs were made using Microsoft Excel 2018 and Graph pad prism 7.00.

5 Results

5.1 Cancer-associated fibroblasts (CAFs) Isolation and Irradiation

CAFs were isolated from non-small-cell lung carcinoma (NSCLC) tissue by enzymatic digestion and outgrowth, as described by *Gorchs et al* [155]. Under light microscope CAFs appeared elongated with spindle-shape morphology as illustrated in *Figure-5.1*. CAFs are anchorage-dependent and grow in a single layer. Culture expanded fibroblasts were checked for lineage specific markers including α-SMA and FAP-1 [156]. In this thesis, we have used CAFs collected from eight different donors. Information on patient demographics as well as tumour stage, size and histologic subtype is summarized in *table 4.1*.

Following initial platting, CAFs were culture expanded for 3 passages before experimentation. At the third passage (3-4 weeks), 70-80 % confluent cultures were irradiated by Varian Clinical linear-accelerator. *Figure 5.1* shows that irradiated cells acquired a flat and enlarged morphology which is indicative of induction of cell senescence and growth arrest. In this study, immunomodulatory functions were compared between control CAFs and CAFs irradiated at single high dose (1x18Gy) or fractionated regimens (3x6Gy).

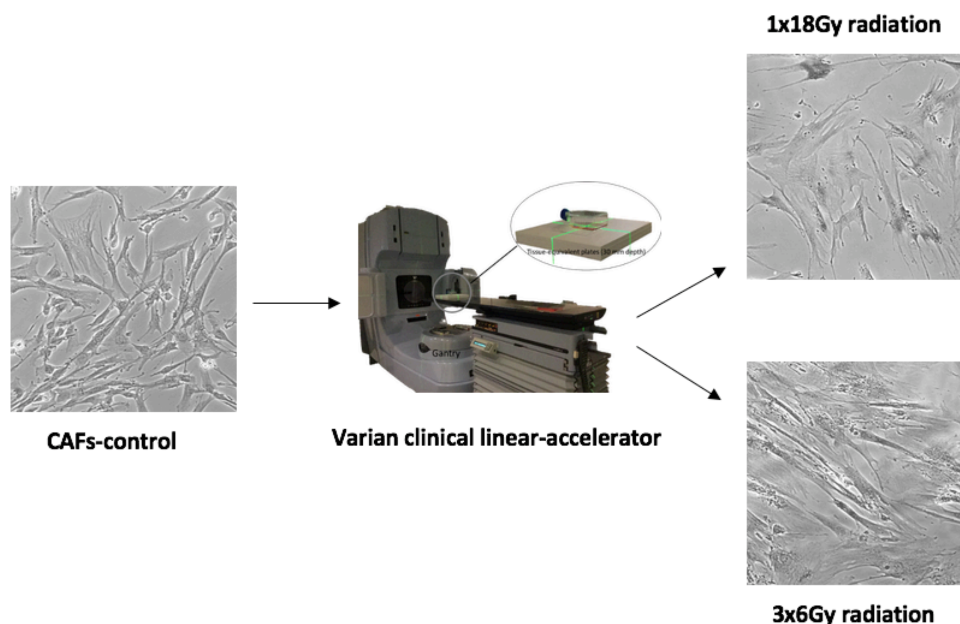


Figure.5.1: CAFs isolation and irradiation: *CAFs were isolated from NSCLC tumor samples, harvested and grown in the lab for 3 passages (70-80% confluency). CAFs were divided into three groups and irradiated by Varian clinical linear-accelerator. One group was irradiated once with a high dose (1x18 Gy) radiation and other group was irradiated with lower fractioned doses three-times 6Gy radiation given in three daily consecutive fractions.*

5.2 Macrophage Isolation and Polarization

Monocytes (CD14+) were isolated from PBMCs (Peripheral blood mononuclear cells) by the use of antibody-coated magnetic beads (MACS). Monocytes appeared circular in shape and attached loosely to plastic. These purified monocytes were incubated six days under the stimulus of M-CSF (100 ng/mL) to promote differentiation into macrophages, here referred to as M0-macrophages.

After six days of incubation, uncommitted M0-macrophages appeared elongated in shape and firmly attached to the surface, under light microscope *(Figure 5.2)*. These macrophages (M0) were then stimulated for 48 hours with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype or with IL-4 (50ng/ml) and TGF-ß (10 ng/mL) for the M2 phenotype. Following 48 hours of stimulation, M1 macrophage appeared more elongated in shape and M2 macrophage become more rounded and less adhesive as illustrated in *Figure.5.2*.

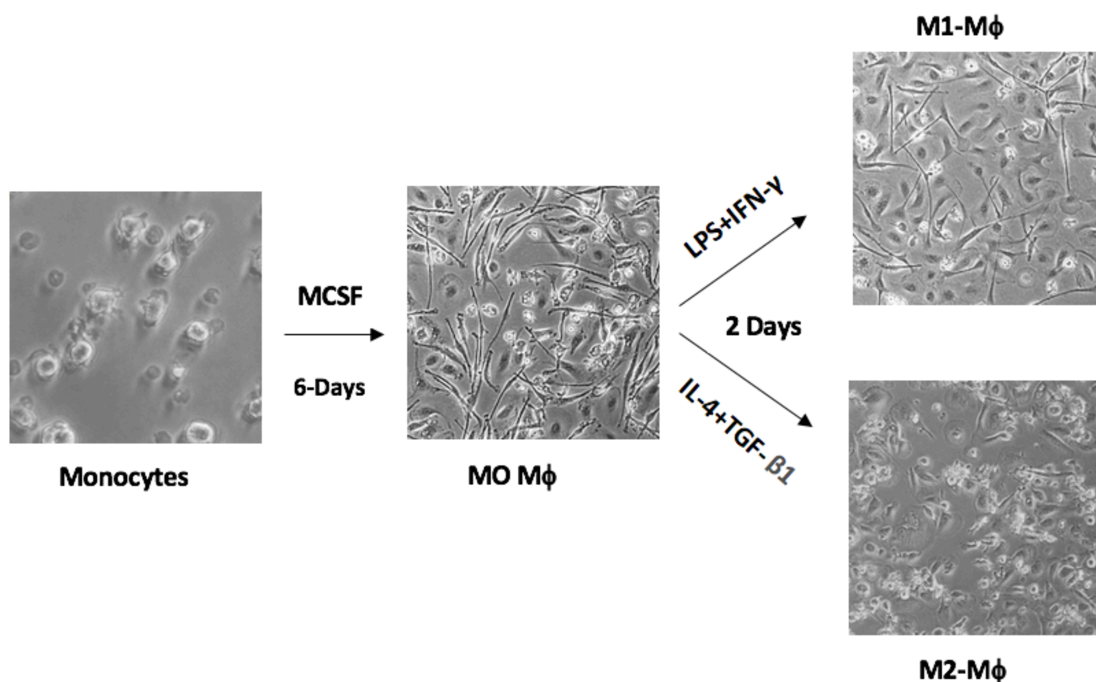


Figure.5.2: *Macrophage isolation and Polarization: Monocytes (CD14+) were isolated from PBMCs (Peripheral blood mononuclear cells). Isolated monocytes were incubated for 6 days with M-CSF to induce macrophage differentiation (Mo-phenotype). Monocyte-derived macrophages were further polarized into M1 and M2 macrophages by LPS+IFN-γ and IL-4+ TGF- ß respectively.*

To characterize macrophage subtypes, we analysed the surface expression of co-regulatory receptors of polarized macrophages. Surface expression of co-regulatory receptors normally overexpressed by M1 macrophages was determined by flow cytometry under different conditions.

Results in *Figure 5.3* show that CD-40, CD-80, and CD-86 are overexpressed after stimulation with LPS and IFN-γ compared to unstimulated (M0) or IL-4+TGF-β stimulated cells (M2).

Unexpectedly, expression of HLA-DR (MHC-II) became down-regulated by LPS and IFN-γ stimulation when compared to unstimulated macrophages. Because of this, we decided to exclude HLA-DR as M1 macrophage marker in our study.

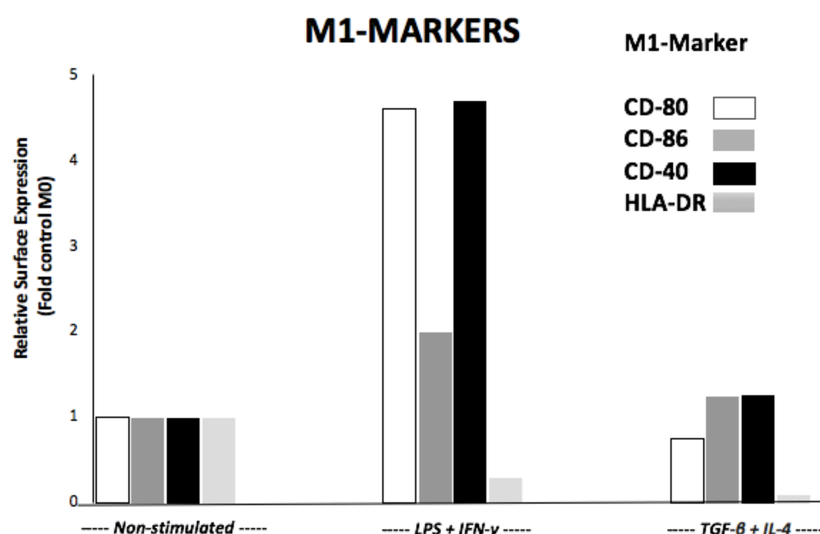


Figure.5.3: Macrophage type-1 markers expression. The expression of CD80, CD86, CD40 and HLA-DR *were analysed on stimulated and unstimulated monocyte-derived macrophages by flow cytometry. Macrophages were stimulated with LPS (100 ng/ml) and IFN-γ (20 ng/ml) for induction of M1 phenotype and with IL-4 (50ng/ml) and TGF- ß (10 ng/ml) for induction of the M2 phenotype.*

Similarly, surface markers normally expressed by the M2 phenotype were determined after stimulation with a different cytokines mix. As shown in *Figure 5.4*, expression of the mannose receptor (CD-206) was elevated in resolution environment (IL-4 and TGF-β stimulation) as compare to M1 (*Figure 5.4*). However, this cytokine cocktail was not able to enhance the expression of the scavenger receptor (CD-163). Inverse results in the expression of CD-206 and CD-163 were observed by stimulation of M0 macrophages with the anti-inflammatory agent dexamethasone (results not shown).

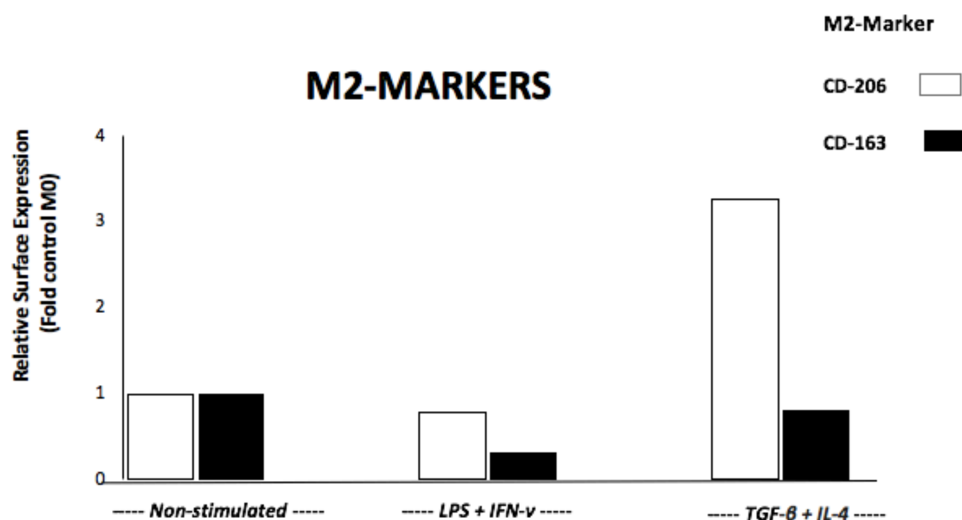


Figure.5.4 Macrophage M2 Cell Surface Marker: The expression of CD-206, and CD-163 *were analysed on monocytes-derived macrophages by flow cytometry. Macrophages (M0) were stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) to induce M1 phenotype and with IL-4 (50ng/mL) and TGF-ß (10 ng/mL) to induce M2 phenotype.*

5.3 Effect of CAFs-CM on Macrophage Polarization

We performed *in vitro* functional assays to investigate whether CAFs-CM (conditioned medium) exerts immune regulatory capabilities and has the power to affect the polarisation of macrophages. Conditioned medium collected from three randomly selected CAF donors exerted different, sometimes opposite, effects on the expression of M1 surface markers from both M1-stimulated and non-stimulated (M0) macrophages (*Figure 5.5-5.7*). While conditioned medium from donor 1 (CAF-CM1) was able to potently induce the expression of the three co-stimulatory receptors on M0 macrophages, the conditioned medium from donors 2 and 3 were only able to enhance the expression of CD-40 (*Figures 5.5-5.7*) in non-stimulated conditions. On the contrary, conditioned medium from donor 3 (CAF-CM3) was able to down regulate the expression of the 3 surface receptors from stimulated (M1) macrophages (*Figures 5.5-5.7*), while the conditioned medium from donor 1 had a modest enhancing effect of the three receptors on M1 macrophages, and CM from donor 2 exerted down regulation CD-40 on stimulated macrophages.

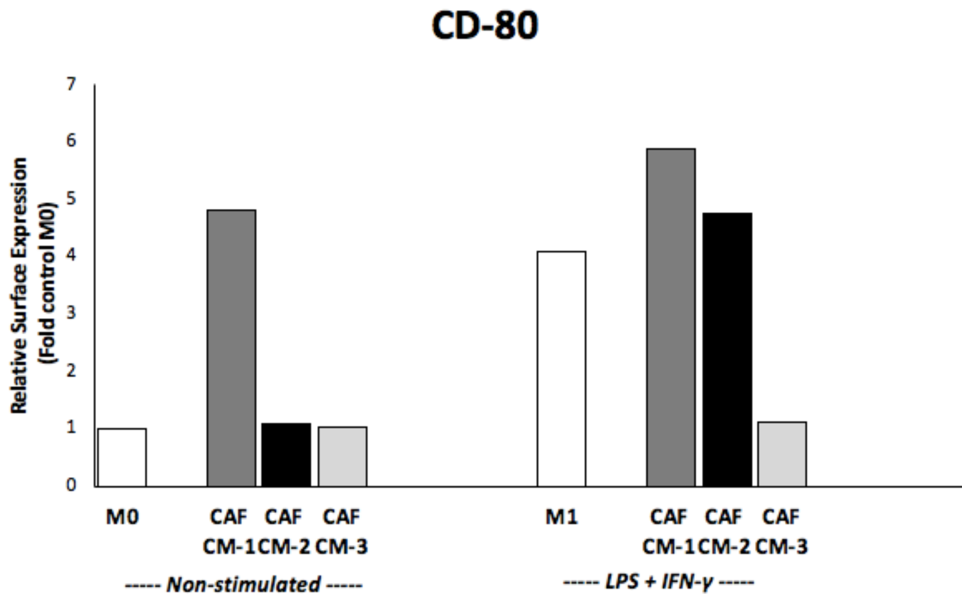


Figure.5.5: Effect of conditioned medium from 3 different CAF donors on the expression of CD-80 on macrophages. *Monocyte-derived macrophages, non-stimulated (M0) and stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype were incubated with CAF-CM for 48 hours, and the expression of CD80 was analysed by flow cytometry. White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls.*

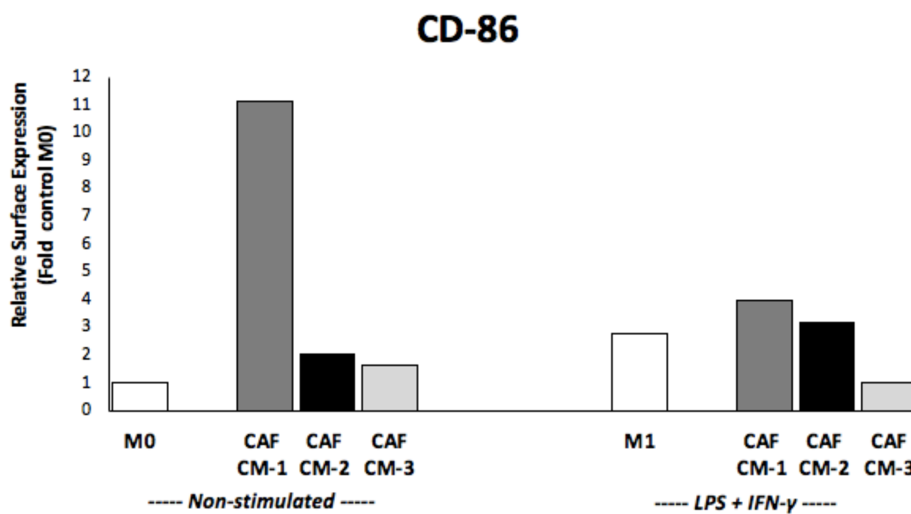


Figure.5.6: Effect of conditioned medium from 3 different CAF donors on the expression of CD-86 on macrophages. *Monocyte-derived macrophages, non-stimulated (M0) and stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype were incubated with CAF-CM for 48 hours, and the expression of CD-86 was analysed by flow cytometry. White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls.*

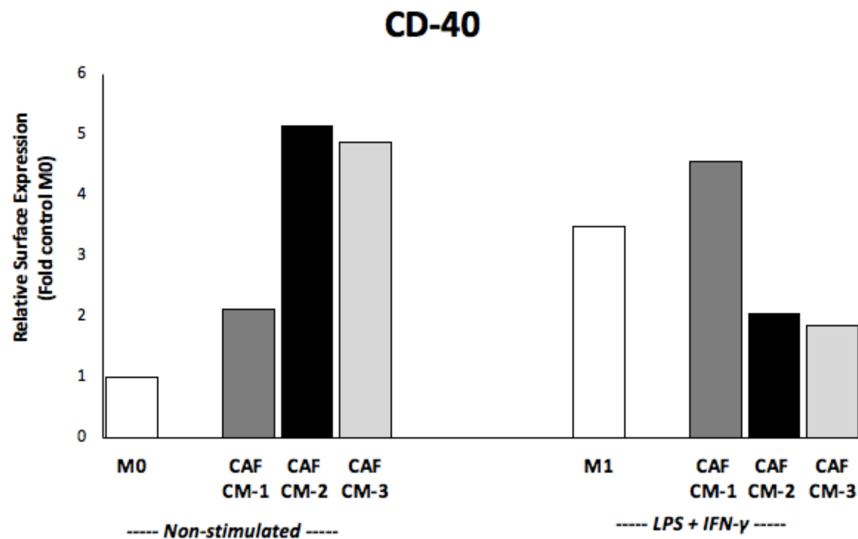


Figure.5.7: Effect of conditioned medium from 3 different CAF donors on the expression of CD-40 on macrophages. *Monocyte-derived macrophages, non-stimulated (M0) and stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype were incubated with CAF-CM for 48 hours, and the expression of CD-40 was analysed by flow cytometry. White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls.*

In a similar manner, effect of CAF-CM on M2 surface markers expression was studied in non-stimulated (M0) and stimulated (M1) conditions. Conditioned medium from the three randomly selected CAF donors were able to induce the expression of the M2 marker CD-206 on non-stimulated macrophages (*Figure 5.8).* However, only conditioned medium from donor 3 (CAF-CM3) was able to induce the same receptor on M1 stimulated macrophages (*Figure 5.8*).

Moreover, conditioned medium from donors 1 and 3 were able to enhance the expression of CD-163 on non-stimulated macrophages, while conditioned medium from donor 2 (CAF-CM2) was decreasing it (*Figure5.9*). Additionally, only conditioned medium from donor 3 (CAF-CM3) was able to induce a relevant enhancement of CD-163 expression if macrophages were stimulated with IFN-γ and LPS.

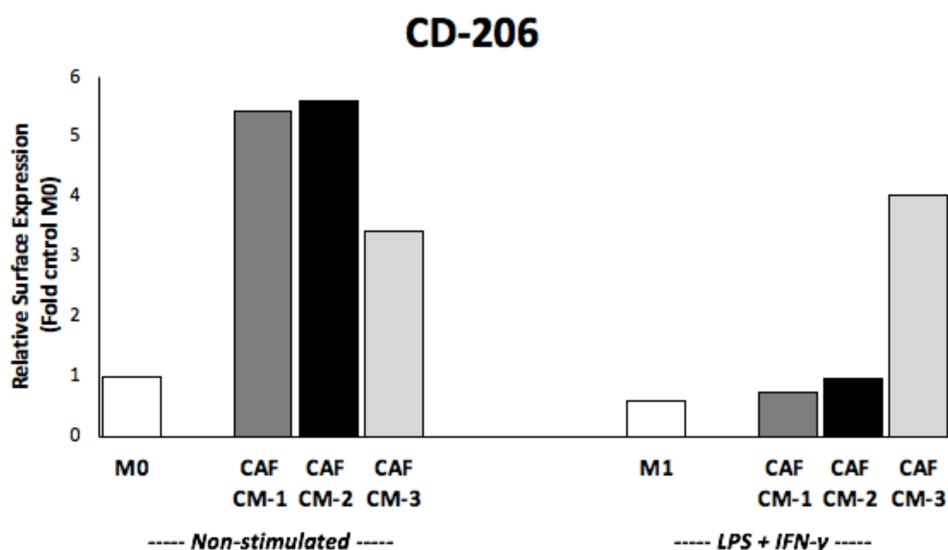


Figure.5.8: Effect of conditioned medium from 3 different CAF donors on the expression of CD-206 on macrophages. *Monocyte-derived macrophages, non-stimulated (M0) and stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype were incubated with CAF-CM for 48 hours, and the expression of CD206 was analysed by flow cytometry. White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls.*

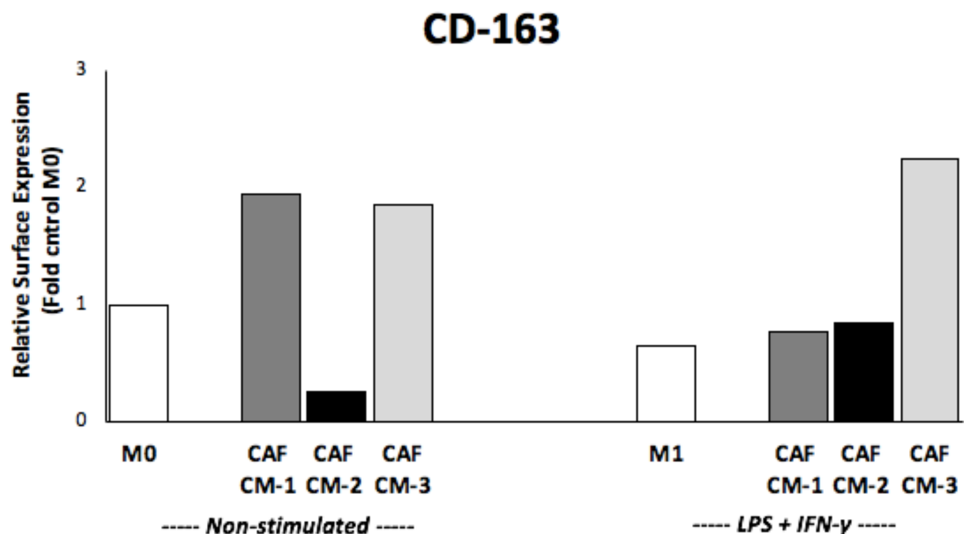


Figure.5.9: Effect of conditioned medium from 3 different CAF donors on the expression of CD-163 on macrophages. *Monocyte-derived macrophages, non-stimulated (M0) and stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype were incubated with CAF-CM for 48 hours, and the expression of CD-163 was analysed by flow cytometry. White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls.*

5.4 Differential effects of irradiated and non-irradiated CAF-CM on Macrophage polarization (Cell Surface Markers)

To analyse the effects of ionizing radiation on CAFs-mediated regulation of macrophages polarisation, we incubated macrophages with CM from both irradiated and non-irradiated CAFs and determined the expression of specific M1 and M2 macrophage surface markers. Of importance, we also investigated the effect of low dose fractioned radiation (three multiple doses of 6 Gy radiation-3x6 Gy) vs. single high dose radiation (one single dose 18 Gy radiation-1x18 Gy) on the cell surface marker of macrophage. In each experiment, 400,000 macrophages/well were incubated for 48 hours with 500 μL of CAF-CM from both irradiated and non-irradiated CAFs (450,000 cells/mL of CM). Results are average determinations from five different CAFs donors.

Neither irradiated nor non-irradiated CAF-CM was able to alter the expression of the co-stimulatory receptor CD-86 in resting (M0) or LPS/ IFN-γ stimulated (M1) conditions (*Figure 5.11*). Very modest enhancing effects were observed on the expression of CD-80. Because of the very low intragroup variability, expression of CD-80 was statistically enhanced in uncommitted (M0) macrophages by all CAF-CM, but unchanged in stimulated (M1) macrophages (*Figure 5.10*).
On the other hand, expression of CD-40 was moderately increased by all CAF-CM in M0 macrophages, but it was statistically significant for both irradiated conditions only. No effects were observed for CD-40 expression in M1-stimulation conditions (*Figure 5.12*).

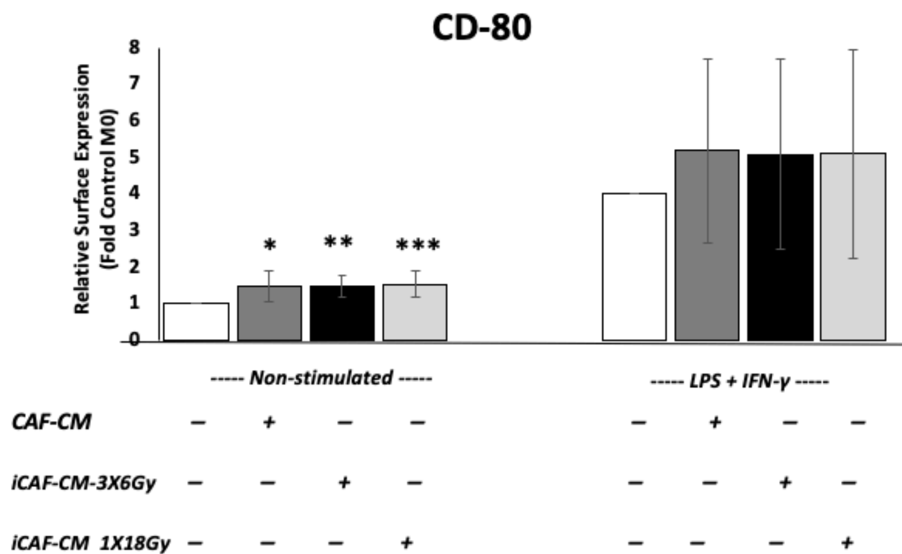


Figure. 5.10: Effect of irradiated and non-irradiated CAF-CM on the expression of cell surface marker CD-80 on macrophage: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of five different CAFs donor from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (*p < 0.0001). iCAF (irradiated CAFs).*

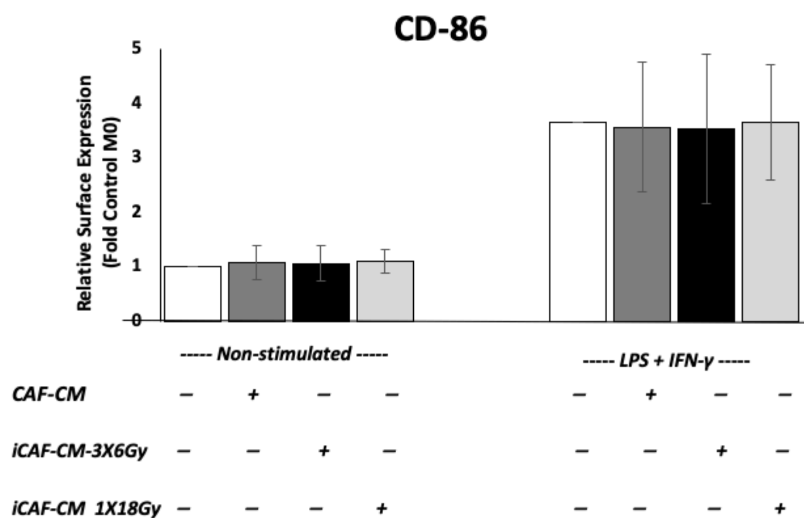


Figure.5.11: Effect of irradiated and non-irradiated CAF-CM on the expression of cell surface marker CD-86 on macrophage: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of five different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (*p < 0.05). iCAF (irradiated CAFs).*

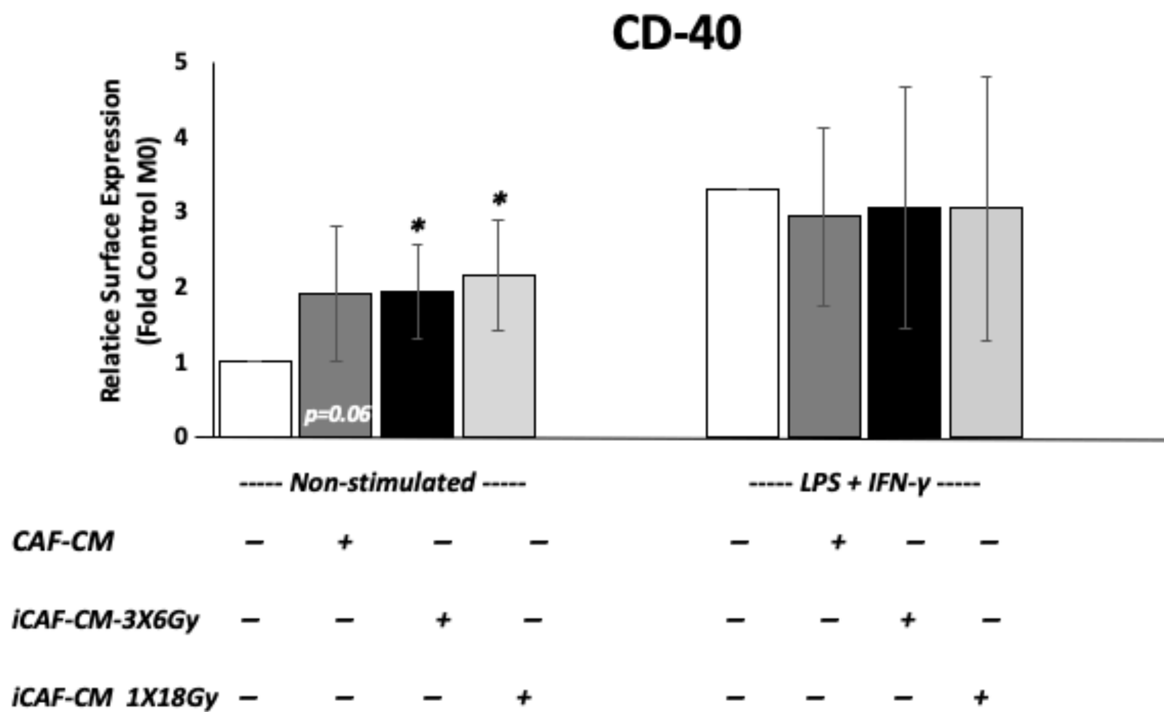


Figure.5.12: Effect of irradiated and non-irradiated CAF-CM on the expression of cell surface marker CD-40 on macrophage: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of five different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (*$p < 0.05$). iCAF (irradiated CAFs).*

Results in *Figure 5.13* show effects of CAF-CM in the expression of CD-206 on macrophages. In non-stimulated conditions (M0), incubation with CAF-CM significantly increases the expression of CD-206 marker by both non-irradiated and irradiated conditions. On the contrary, in LPS and IFN-γ stimulated condition, the two irradiated CAF-CM slightly decrease the expression of CD-206 markers expression. *Figure 5.14* shows that expression of CD-163 increases to some extent after incubation of macrophages with irradiated and non-irradiated CAF-CM, even though differences are not significant. Similarly, in LPS and IFN-γ stimulated conditions, both irradiated CAF-CM slightly increase the expression of CD-163. No statistical differences were observed between the irradiated and non-irradiated CAF-CM.

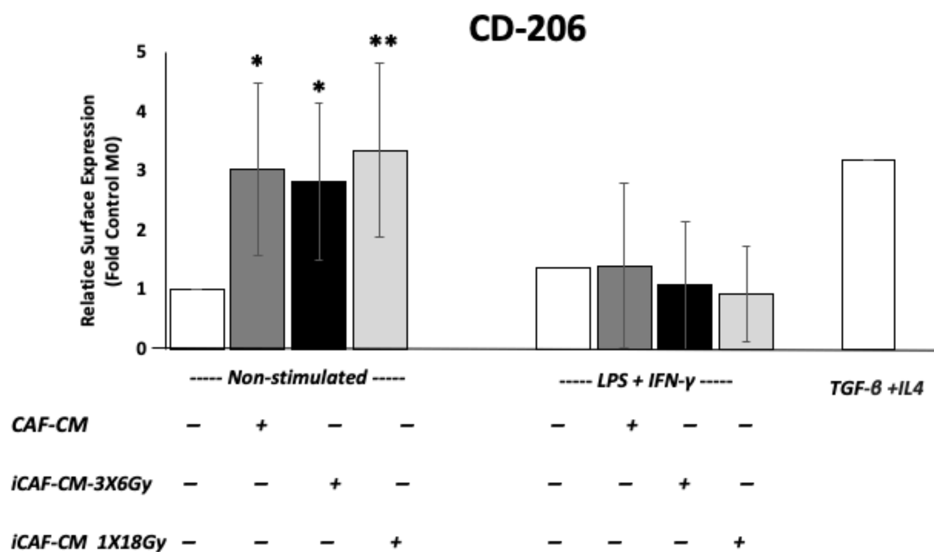


Figure. 5.13: Effect of irradiated and non-irradiated CAF-CM on the expression of cell surface marker CD-206 on macrophage: *White bars indicate expression levels in control macrophage cultures (M0, M1 and M2). Results are expressed as fold M0 controls. Data represent the mean values of five different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (**$p < 0.001$). iCAF (irradiated CAFs).*

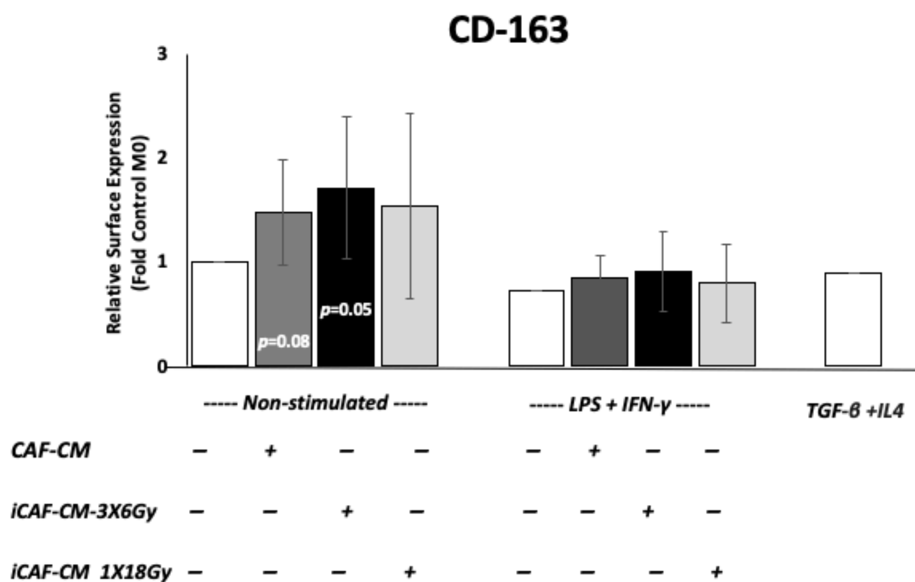


Figure. 5.14: Effect of irradiated and non-irradiated CAF-CM on the expression of cell surface marker CD-163 on macrophage: *White bars indicate expression levels in control macrophage cultures (M0, M1 and M2). Results are expressed as fold M0 controls. Data represent the mean values of five different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (*$p < 0.05$). iCAF (irradiated CAFs).*

5.5 Effect of CAF-CM on Macrophage-derived Nitric Oxide production

To further investigate the paracrine effect of CAF-CM, we performed Nitric oxide assay by using the fluorescent probe diaminofluorescein diacetate (DAF-2DA).

Outcomes of the control experiment is shown in *Figure 5.15*. Nitric oxide production (NO) was higher in pro-inflammatory conditions (LPS and IFN-γ stimulation). Inversely, in resolving conditions with TGF-β and IL-4, the production of NO is even lower than in non-stimulated conditions (*Figure 5.15*).

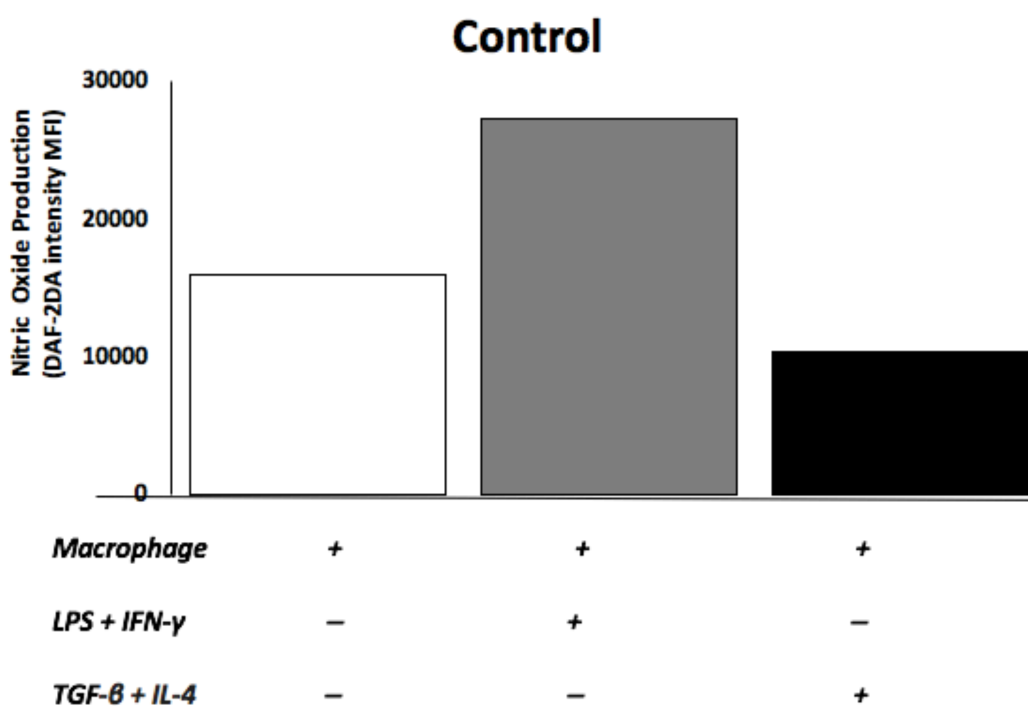


Figure. 5.15: Nitric oxide production by stimulated and non-stimulated macrophages: *Median fluorescence intensity by DAF-2DA for Monocyte-derived macrophages non-stimulated (M0), stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for M1 phenotype and stimulated with IL-4 (50ng/mL) and TGF- β (10 ng/mL) for M2-phenotype was analysed by flowcytometry. Nitric oxide production was high in M1 phenotype as compared to M0 and M2 states. DAF-2DA (4,5-Diaminofluorescein diacetate).*

Conditioned media from all CAF experimental groups could slightly increase the production of NO by uncommitted macrophages, although results were significant for 1x 18 Gy irradiated CAF-CM only *(Figure 5.16)*. On the other hand, significant reduction in the production of nitric oxide was found in M1 stimulated macrophages upon incubation with both irradiated and non-irradiated CAF-CM. No differences were observed between the irradiated and non-irradiated CAF-CM.

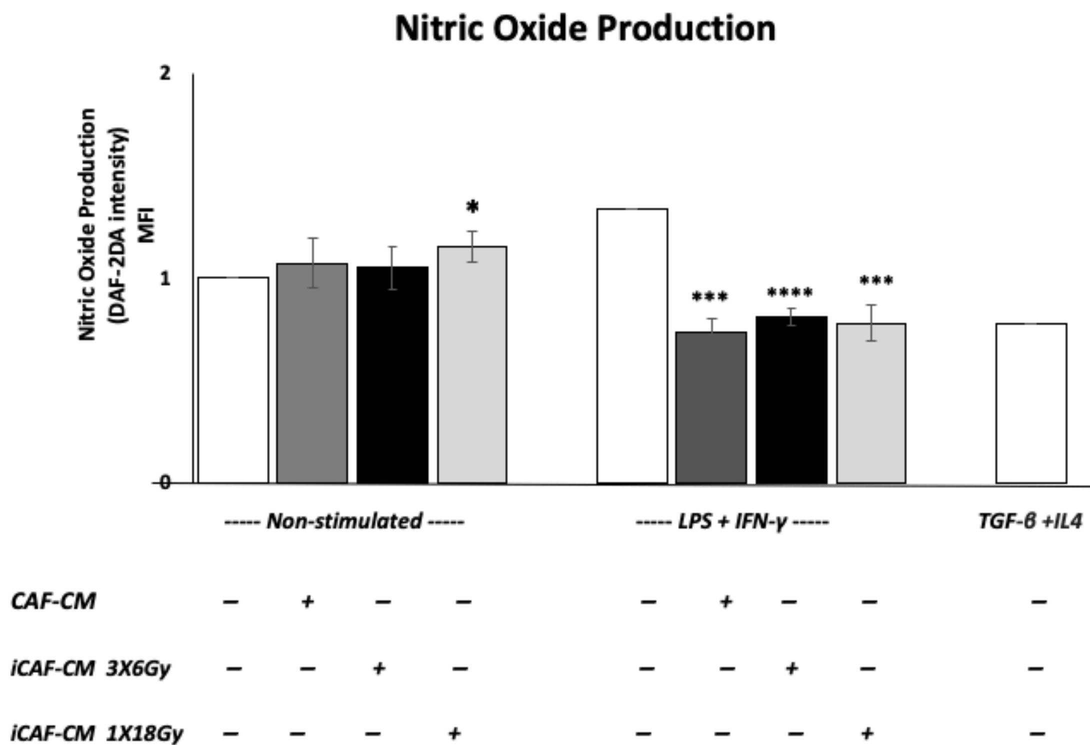


Figure. 5.16: Effect of irradiated and non-irradiated CAFs on the production of Nitric oxide in polarized macrophages by flow cytometry: *White bars indicate expression levels in control macrophage cultures (M0, M1 and M2). Results are expressed as fold M0 controls. Data represent the mean values of three different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (****$p < 0.0001$). DAF-2DA (4,5-Diaminofluorescein diacetate). iCAF (irradiated CAFs).*

5.6 Effect of CAFs-CM on Cytokine Expression by Macrophages

As an alternative way to measure macrophage phenotype polarization, we proceeded to quantify pro-inflammatory and anti-inflammatory cytokines secreted to the media by macrophages, in the presence or absence of irradiated and non-irradiated CAFs-CM.

First we proceeded to check the expression of cytokines secreted by CAFs themselves. To reproduce the same experimental conditions, CAF cells were irradiated with single high-dose (1x18 Gy and fractionated low dose (3x6 Gy) in a 24 well plate ($0.2x10^6$ CAFs/well) and incubated for 48 hours at 37ºC in a 5 % CO_2 humidified atmosphere. After that, supernatants were collected to quantify the pro-inflammatory (IL-6, IL-12 and TNF-a) and anti-inflammatory (IL-10) cytokines. Determination of human IL-6, TNF-α, IL-12, and IL-10 was done by specific ELISA immune assays.

Figure 5.17 shown that CAFs secretes some amounts of IL-6 (300 pg/mL), while IL-12, IL-10 and TNF- α were not detected on CAFs supernatant. Values corresponding to CAF-derived IL-6 were substracted from the total values obtained on macrophage cultures.

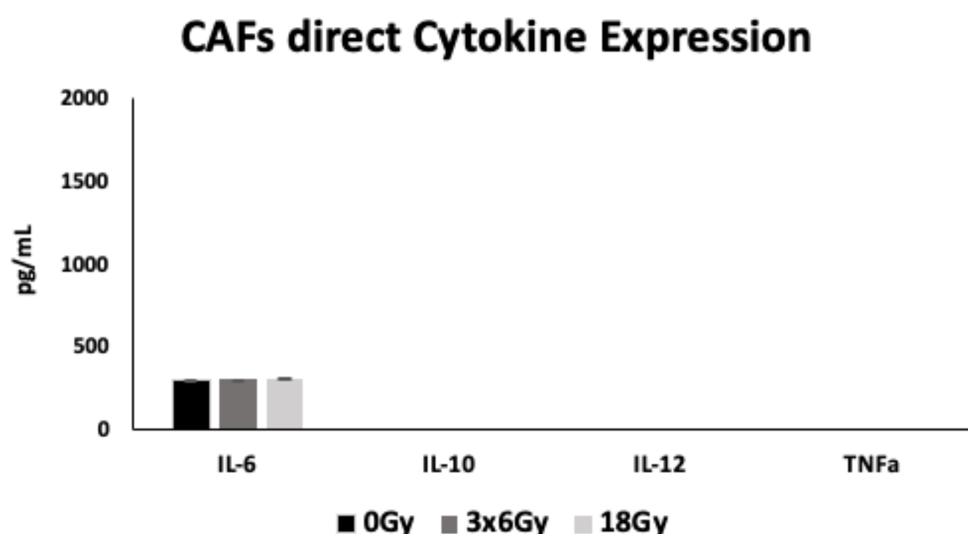


Figure. 5.17: *Pro-inflammatory and anti-inflammatory cytokine secretion by CAFs, quantified by ELISA essay.*

Control experiment for expression of IL-6 by macrophages is shown in *Figure 5.18*. As expected, production of IL-6 is increased in pro-inflammatory conditions (LPS + IFN-γ) and decreased to baseline levels under resolving conditions (TGF-β + IL-4)

When incubated with CAF-CM, all CM were able to increase to a certain degree IL-6 production by both non-stimulated, and LPS/IFN-γ stimulated macrophages (*Figure 5.19*). In non-stimulated macrophages, the result was statistically significant only for low dose fractioned (3x6 Gy) radiation. On the contrary, in LPS and IFN-γ stimulated condition, it was significant only for the non-

irradiated CAF-CM. No statistically significant differences were observed in both irradiated CAF-CM due to larger inter-donor variabilities.

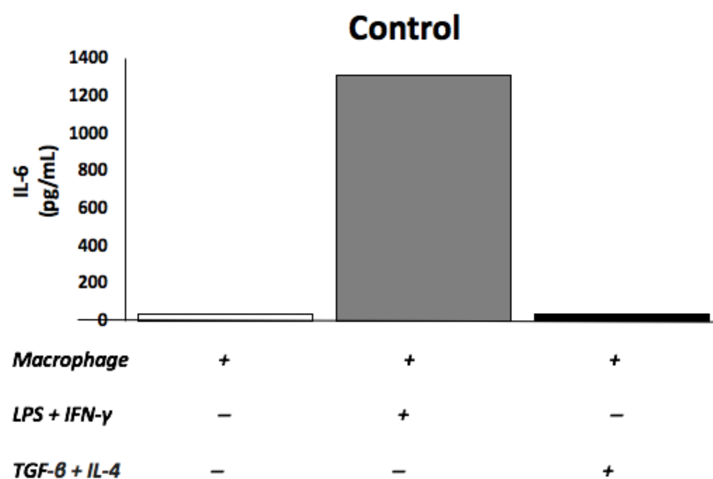


Figure. 5.18: IL-6 production by monocyte-derived macrophages in different stimulatory conditions quantified by ELISA essay. *Results only represent macrophage-derived IL-6 as CAF-derived IL-6 were subtracted from full measurements.*

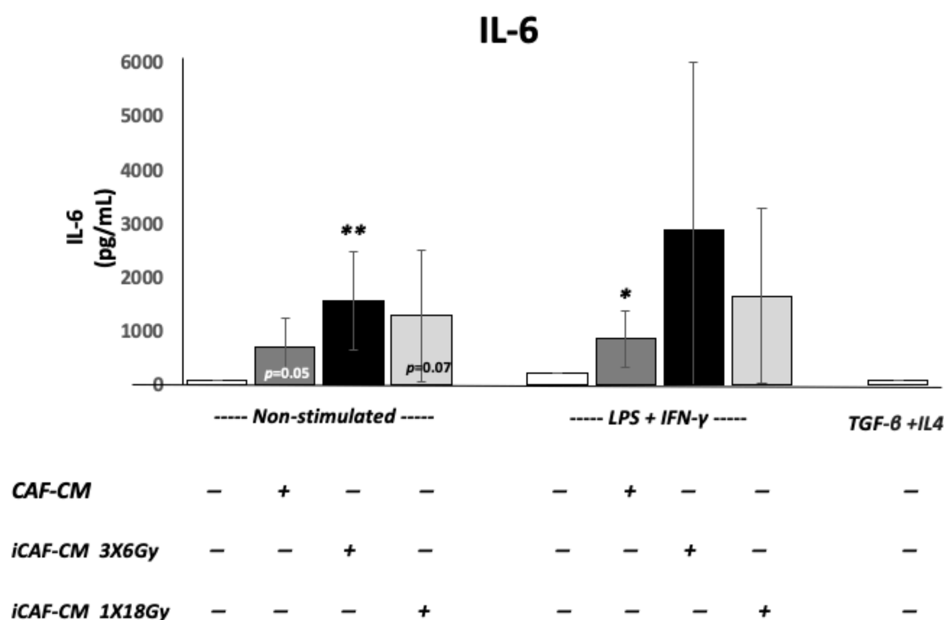


Figure. 5.19: IL-6 produced by monocyte derived macrophages incubated with irradiated or non-irradiated CAF-CM were quantified by ELISA assay. *Data represent the mean ±SD values from five independent experiments measured in duplicates. CAF-derived IL-6 was subtracted from full measurements. Results only represent macrophage-derived IL-6 as CAF-derived IL-6 were subtracted from full measurements. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM. (**$p < 0.001$). iCAF=Irradiated CAFs.*

Similarly, *Figure 5.20* shows the TNF-α production by macrophages in control experiments. TNF-α levels were high in the LPS and IFN-γ stimulated condition, while it was minimal in non-stimulated and resolving condition with IL-4 and TGF-ß condition as expected. Of note, very low levels of TNF-α were measured in supernatants from irradiated and non-irradiated CAFs.

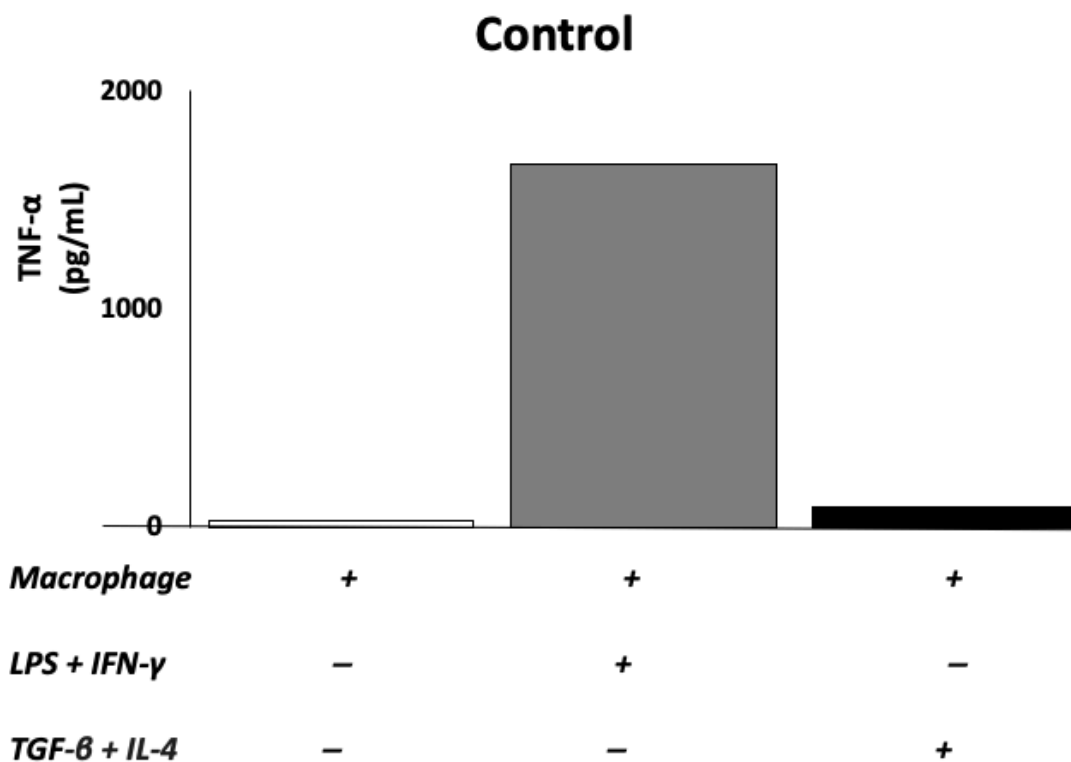


***Figure. 5.20: TNF-α** production by monocyte derived macrophages in different stimulatory conditions measured by ELISA assay.*

In experiments with CAF-CM, none of the experimental groups was able to modulate TNF- α expression by M0 macrophages, however, CM from both irradiated and non-irradiated CAF were able to decrease the production of TNF- α in M1 polarized macrophages. The result was statistically significant for non-irradiated and 3x6 Gy irradiated CAF-CM (*Figure 5.21).*

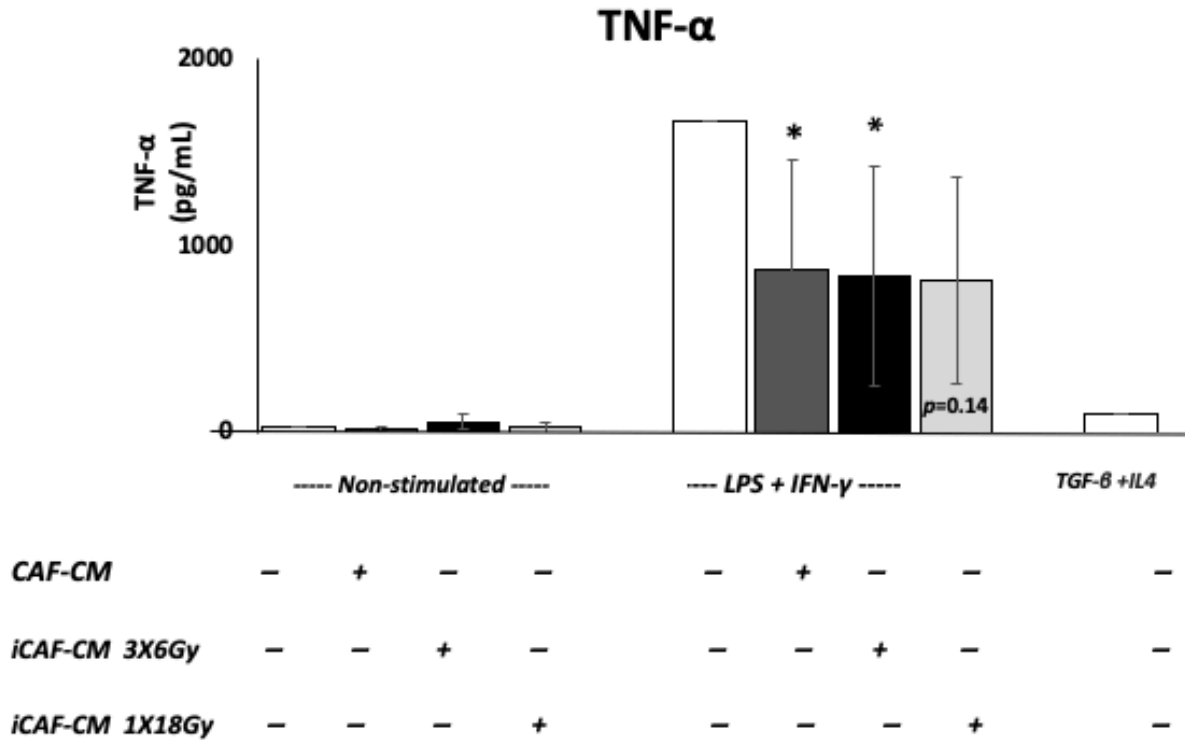


Figure.5.21: TNF-α produced by monocyte derived macrophages incubated with irradiated or non-irradiated CAF-CM were quantified by ELISA assay. *Data represent the mean ±SD values from five independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (*$p < 0.05$). iCAF=Irradiated CAFs.*

IL-12 is another proinflammatory cytokines secreted by macrophages. In control experiments, we clearly observe high production of IL-12 in LPS and IFN-γ stimulated condition. While, the production of IL-12 was undetectable in non-stimulated or IL-4 and TGF-ß stimulated macrophages (*Figure 5.22*) displayed on next page.

In CAF-CM experiments, we observe that CM from all experimental groups significantly block the production of IL-12 by M1-polarized macrophages (*Figure 5.23*) displayed on next page.

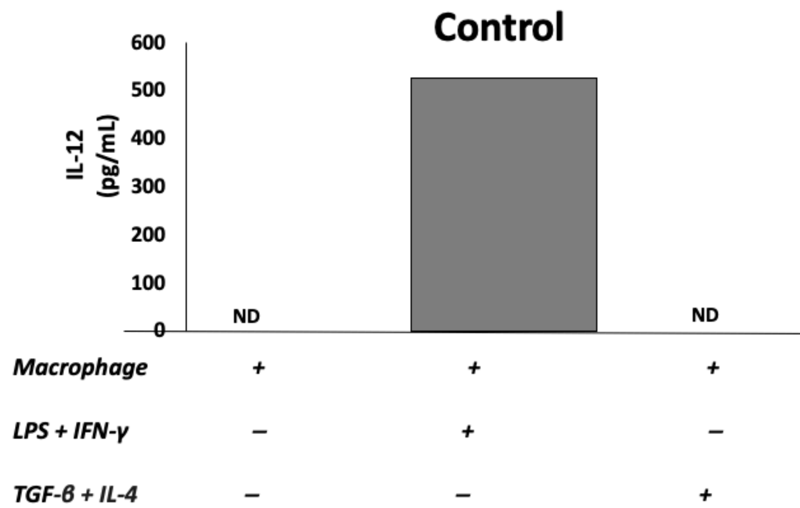


Figure.5.22: IL-12 production by monocyte derived macrophages in different stimulatory conditions measured by ELISA assay. *ND. Non-detected.*

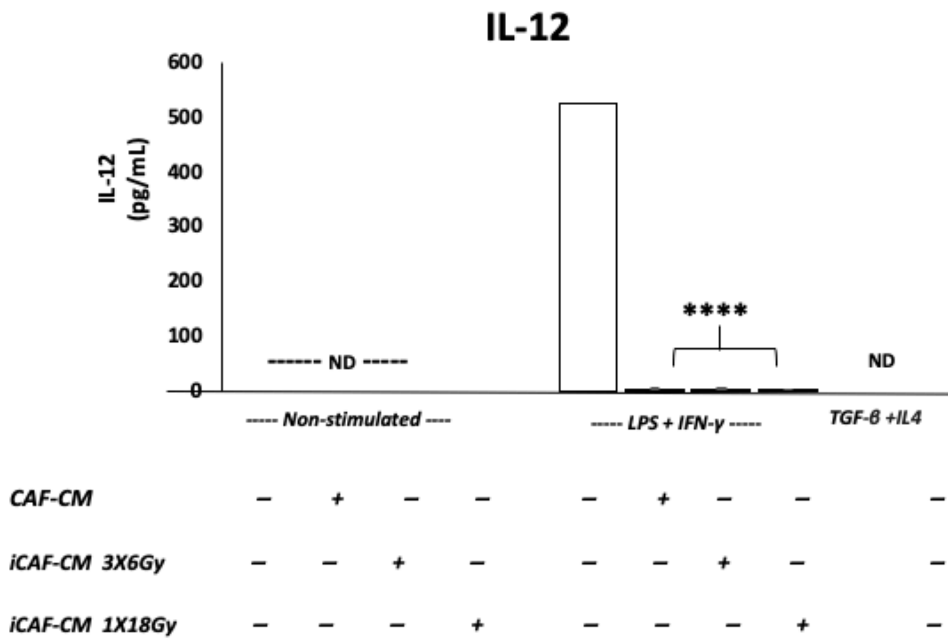


Figure.5.23: IL-12 cytokine produced by monocyte derived macrophages incubated with irradiated or non-irradiated CAF-CM were quantified by ELISA assay. *Data represent the mean ±SD values from five independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM individually. (****$p < 0.0001$).ND. Non-detected. iCAF=Irradiated CAFs.*

Interleukin (IL)-10 is a recognized marker of M2 polarized macrophages [2] and supposed to be induced in the IL-4 and TGF-ß stimulated condition. Unexpectedly, the production of IL-10 was highest in the LPS+IFN-γ stimulated condition (*Figure 5.24*). Previous studies have also reported similar observations with IL-10 [157]. As internal control for the assay, we checked IL-10 measurements in samples containing exogenously added recombinant IL-10 (result not shown). The latter experiments confirmed the correct functioning of the ELISA assay.

The conditioned medium from all CAF experimental groups were able to trigger significantly high expression of IL-10 by uncommitted macrophages. On the other hand, both irradiated and non-irradiated CAF-CM, slightly reduced the IL-10 production in M1-polrized macrophages. However, the result was significant only for non-irradiated CAF-CM.

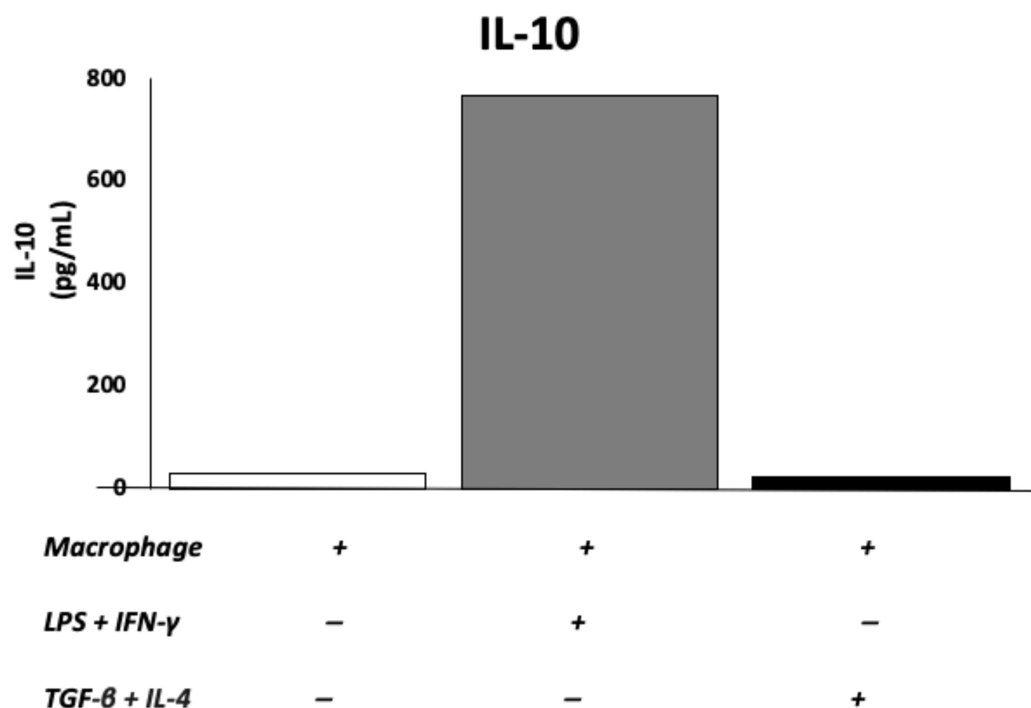


Figure.5.24: *IL-10 production by monocyte derived macrophages in different stimulatory conditions, measured by ELISA assay.*

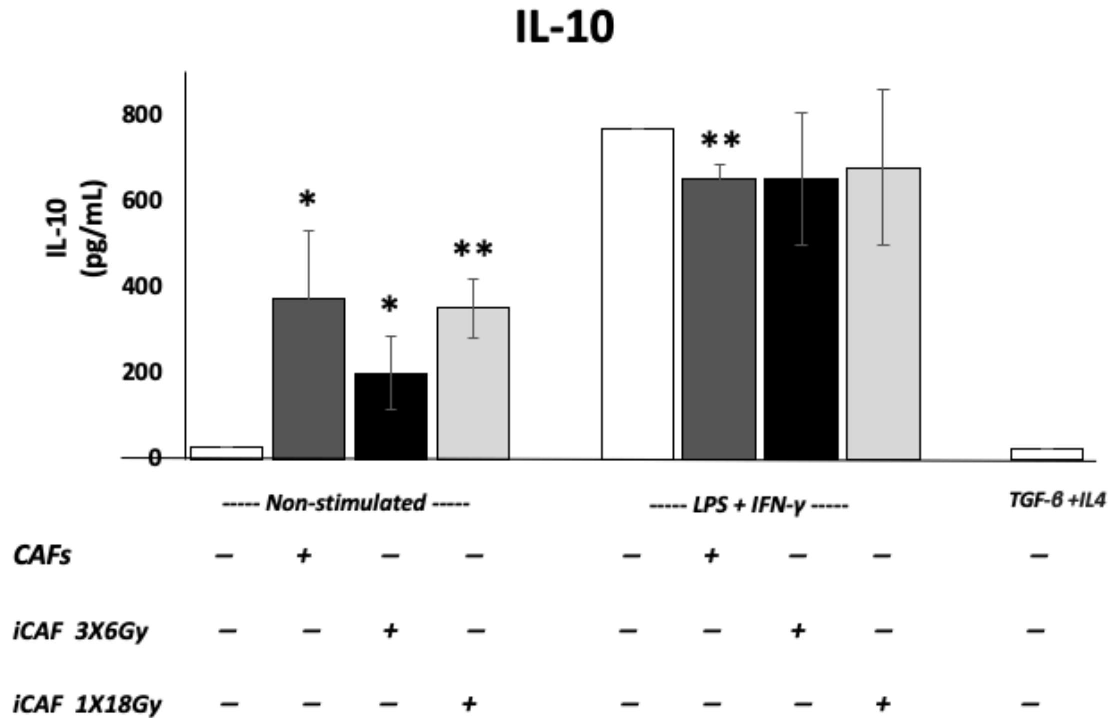


Figure.5.25: IL-10 cytokine produced by monocyte derived macrophages incubated with irradiated or non-irradiated CAF-CM were quantified by ELISA assay. *Data represent the mean ±SD values from five independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF-CM, control and both irradiated CAF-CM. (**$p < 0.001$). iCAF=Irradiated CAFs.*

5.7 CAF-mediated effects on Macrophage cell surface markers during co-culture.

To ascertain if CAFs could mediate additional or stronger effects on macrophages via direct cell-cell interactions, we proceeded to measure effects in co-culture conditions. In each experiment, 400,000 cells/well macrophages were co-cultured for 48 hours with 200,000 x10^6 cells/well of CAFs (both irradiated and non-irradiated). CAF cultures were used for co-culture experiments 24 hours after the last radiation cycle. Macrophages from co-cultures with fibroblasts were positively selected by CD-45$^+$ expression on flow cytometry as shown in *Figure 5.26*.

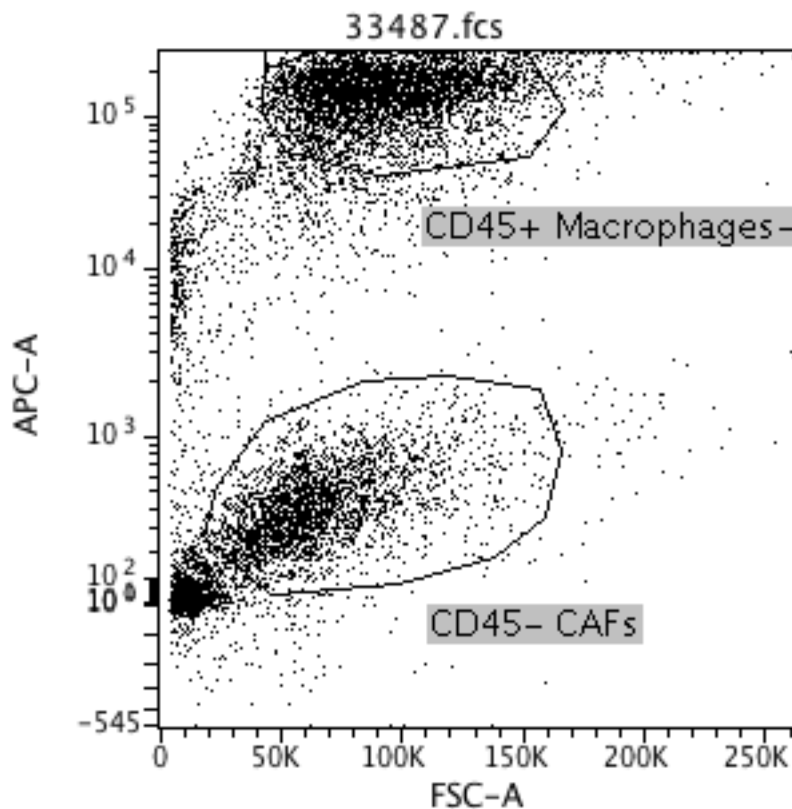


Fig. 5.26. Flow cytometry gate data. *Macrophages from co-culture with fibroblasts were positive selected by CD-45⁺ expression (APC fluorochrome) and the fibroblast was mentioned as CD-45⁻ population.*

CAFs in co-culture with uncommitted macrophages (M0) were able to induce a slight increase of CD-80 but had no effects on the expression of CD-86 or CD-40, with the exception of the 3x6 Gy irradiated CAFs group which had an inductive effect on the expression of CD-40 (*Figures 5.27-5.29)*. Otherwise, no big differences were observed between irradiated and non-irradiated CAFs receptor regulation in any of the experimental groups.

CAFs in co-cultures with M1-stimulated macrophages induced a slight decrease of CD-80 expression and a significant decrease of CD-40 expression, while had no effects on CD-86 expression (*Figures 5.27-5.29*). Results were statistically significant for all CAFs group on CD-40 expression levels.

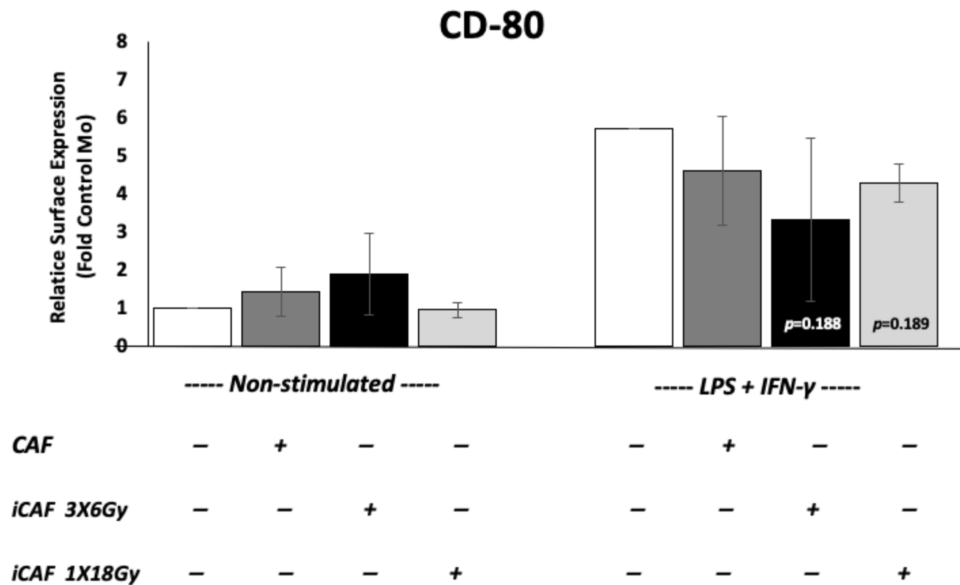


Figure.5.27: Effect of irradiated and non-irradiated CAFs on the expression of cell surface marker CD-80 on macrophages during co-culture: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of four different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated, control and both irradiated CAF individually. (*p< 0.05). iCAF=Irradiated CAFs.*

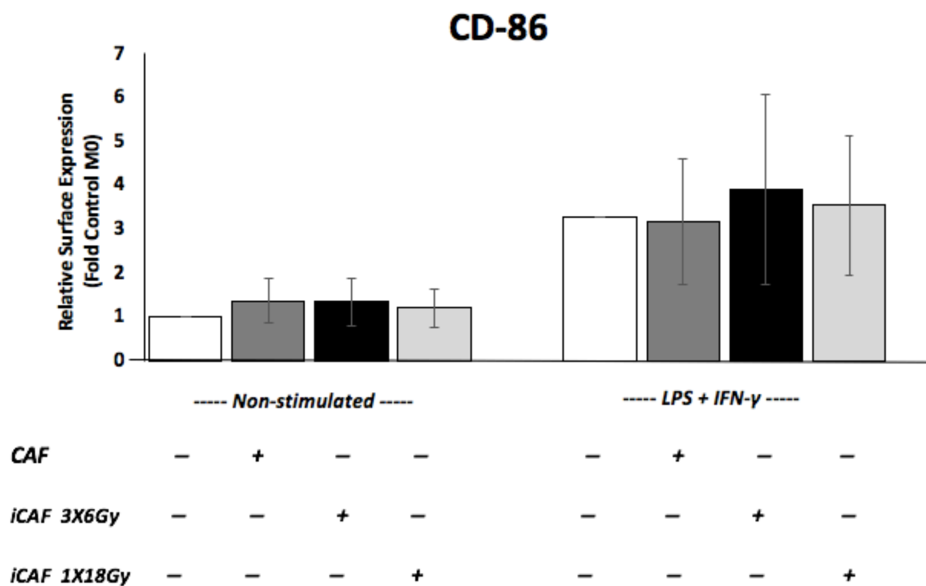


Figure.5.28: Effect of irradiated and non-irradiated CAFs on the expression of cell surface marker CD-86 on macrophages during co-culture: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of four different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF. (*p < 0.05). iCAF=Irradiated CAFs.*

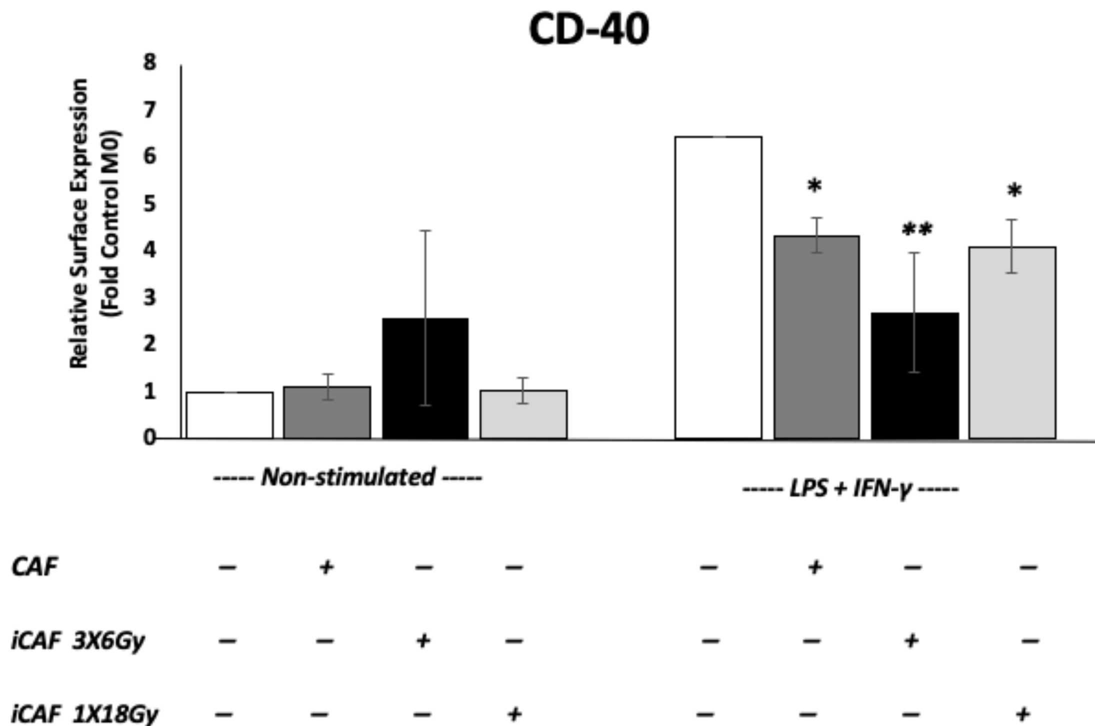


Figure.5.29: Effect of irradiated and non-irradiated CAFs on the expression of cell surface marker CD-40 on macrophages during co-culture: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of four different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF individually. (**$p < 0.001$). iCAF=Irradiated CAFs.*

Non-irradiated CAFs induced a noticeable enhancement in the expression of both M2 markers CD-206 and CD-163 in uncommitted (M0) macrophages, however, this effect was to some extent blocked in the two irradiated CAFs groups *(Figures 5.30-5.31)*. On the other hand, enhanced expression of both CD-206 and CD-163 on M1 stimulated macrophages was observed only when CAFs were irradiated at 3x6Gy *(Figures 5.30-5.31)*. However, differences with controls were not statistically significant due to prominent inter-donor heterogeneity.

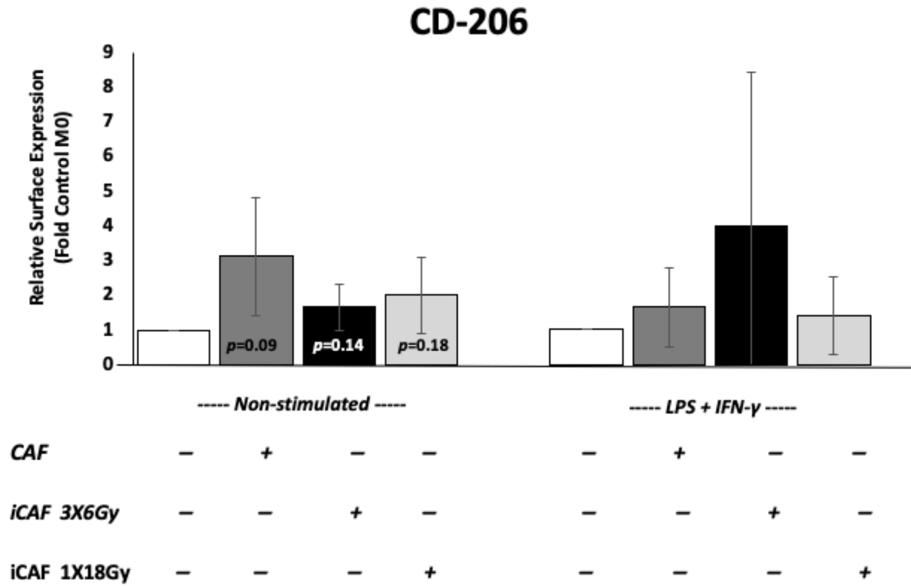


Figure.5.30: Effect of irradiated and non-irradiated CAFs on the expression of cell surface marker CD-206 on macrophages during co-cultures: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of four different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF individually. (*p < 0.05). iCAF=Irradiated CAFs.*

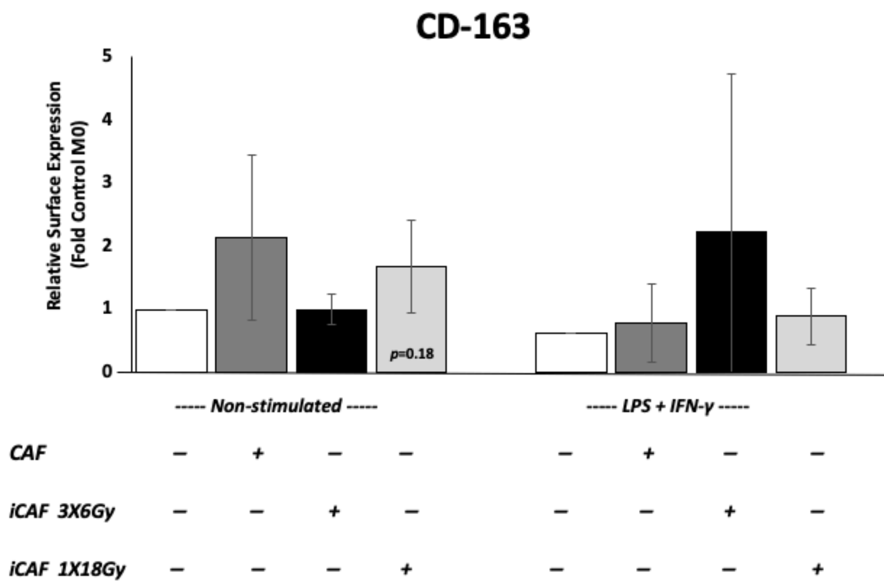


Figure.5.31: Effect of irradiated and non-irradiated CAFs on the expression of cell surface marker CD-163 on macrophages during co-culture: *White bars indicate expression levels in control macrophage cultures (M0 and M1). Results are expressed as fold M0 controls. Data represent the mean values of four different CAFs donors from triplicate determinations. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF individually. (*p < 0.05). iCAF=Irradiated CAFs.*

5.8 Nitric Oxide production by Macrophages during co-cultures with CAFs

In order to measure the immunomodulatory effect of CAFs on nitric oxide production by macrophages, monocyte-derived macrophages were seeded together with irradiated and non-irradiated CAFs cells for 48 hours. Nitric oxide assay was performed by using fluorescent probe diaminofluorescein diacetate DAF-2DA as described earlier. In **control** experiments, we demonstrated that pro-inflammatory conditions (LPS and IFN-γ stimulation) triggered an enhanced production of nitric oxide from macrophages. While its production was lower in non-stimulated (M0) and resolving condition with TGF-β and IL-4 (M2) (*Figure 5.15*).

CAFs from both irradiated and non-irradiated conditions statistically enhance nitric oxide production by M0-macrophages (*Figure 5.32*). On the opposite, all CAFs groups decreased the production of nitric oxide in the LPS- IFN-γ stimulated condition (M1-macrophagres). However, only the group of irradiated CAFs was able to decrease significantly the production of nitric oxide in M1-macrophages (*Figure 5.32*).

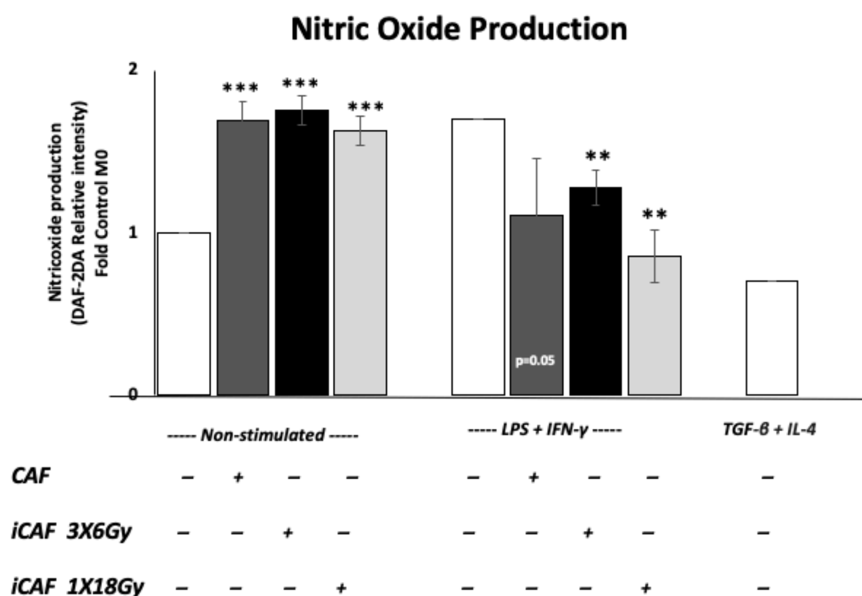


Figure.5.32: *Effect of irradiated and non-irradiated CAFs co-cultures on the production of Nitric oxide by polarized macrophages: Median fluorescence intensity DAF-2DA in Monocyte-derived macrophages non-stimulated (M0), stimulated with LPS (100 ng/ml) and IFN-γ (20 ng/ml) for M1 phenotype, and stimulated with IL-4 (50ng/ml) and TGF- β (10 ng/ml) for M2-phenotype was analysed by flow cytometry. Stimulated and non-stimulated macrophages were co-cultured with irradiated and non-irradiated CAFs for 48 hours. Data represent the mean values of three different CAFs donors from triplicate determinations. Results are expressed as fold M0 controls. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF individually. (***p < 0.0001). DAF-2DA (4,5-Diaminofluorescein diacetate). iCAF=Irradiated CAFs.*

5.9 Effect of CAFs-Co-culture on Cytokines expression by Macrophages

As we did with conditioned media, we checked the effects of irradiated and non-irradiated CAFs co-cultures on the production of IL-6, TNF-α, IL-12 and IL-10 from uncommitted (M0) and LPS/IFN-γ stimulated (M1) macrophages. As shown in *Figure 5.33* control experiments showed elevated secretion of IL-6, IL-12 and TNF-α in LPS/IFNγ stimulated conditions, and negligible amounts produced in un-stimulated or TGFβ/IL-4 stimulated conditions. Unexpectedly, IL-10 was also enhanced during LPS/IFNγ stimulation and unchanged during TGFβ/IL-4 stimulation (*Figure-5.33).*

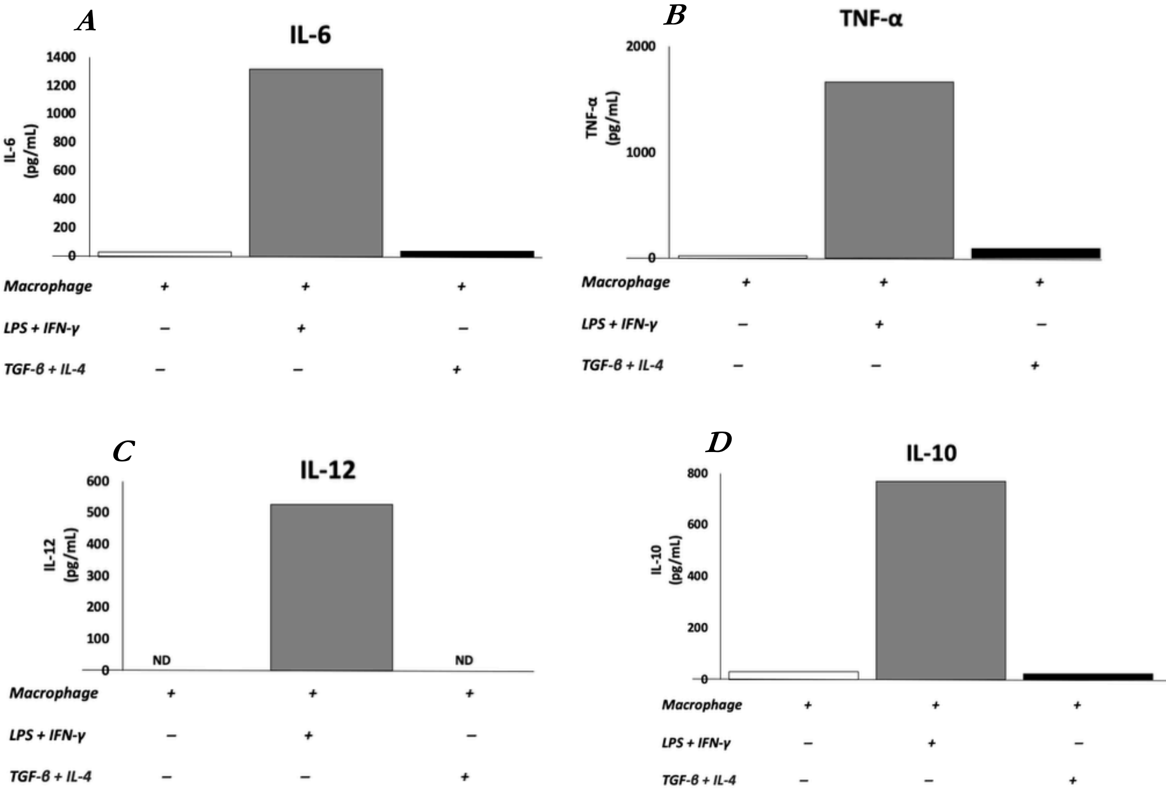


***Figure.5.33**: Cytokine production by monocyte derived macrophages in different stimulatory conditions, measured by ELISA assay. **A**-IL-6, **B**-TNF-α, **C**-IL-12 **D**-IL-10. (This figure is repetition of Figures5.18,5.20, 5.22 and 5.24)*

In the case of non-stimulated macrophages, both irradiated and non-irradiated CAFs were able to enhance IL-6 production. However, results were significant only for the case of non-irradiated and fractionated regimen (3x6 Gy) CAFs. The irradiated group 1x18 Gy could also increase the production of IL-6, but it was not significant (*Figure 5.34*).

On the other side, all CAFs groups increased the production of IL-6 in the LPS- IFN-γ stimulated condition (M1-macrophagres), although the result was significant only for single high dose irradiated (1x18 Gy) CAFs (*Figure 5.34*).

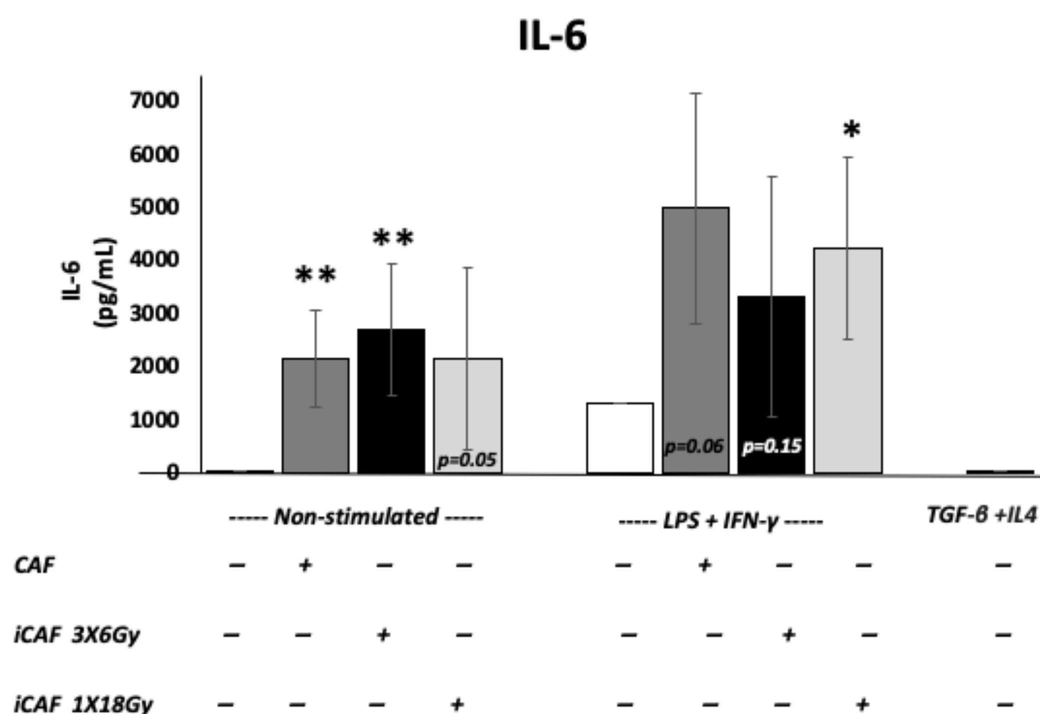


Figure. 5.34: Detection of IL-6 produced by monocyte derived macrophages co-cultured with irradiated or non-irradiated CAFs quantified by ELISA assay. *CAF-derived IL-6 was subtracted from full measurements. Data represent the mean ±SD values from four independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF. (*p < 0.005). iCAF=Irradiated CAFs.*

The production of TNF-α by uncommitted macrophages was negligible and unchanged after co-cultures with irradiated or non-irradiated CAFs. However, irradiated CAFs were able to induce a significant reduction of TNF-α secretion by M1 stimulated macrophages (*Figure 5.35*).

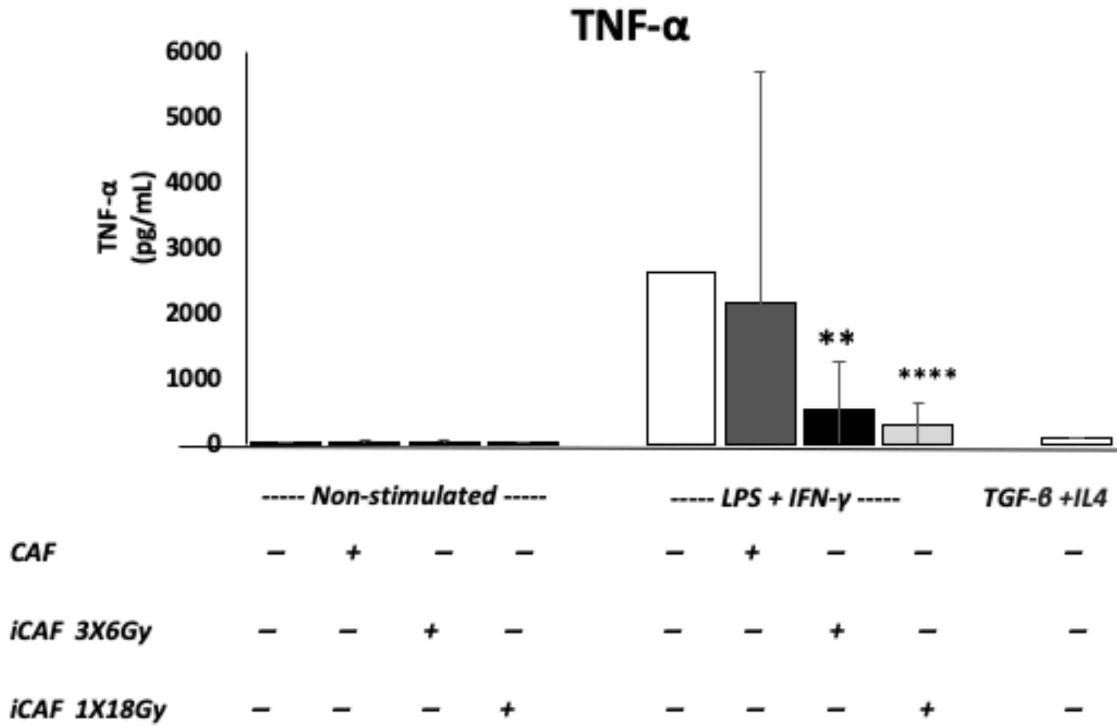


Figure.5.35: Detection of TNF-α produced by monocyte derived macrophages co-cultured with irradiated or non-irradiated CAFs measured by ELISA assay. *Data represent the mean ±SD values from four independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF. (****p < 0.00001). iCAF=Irradiated CAFs.*

Interleukin (IL)-12 was undetectable in the media of uncommitted macrophages and this scenario persisted during co-culture with CAFs (*Figure 5.36*). However, both irradiated and non-irradiated CAFs were able to significantly reduce the baseline levels of IL-12 produced by LPS/IFN- γ stimulated macrophages.

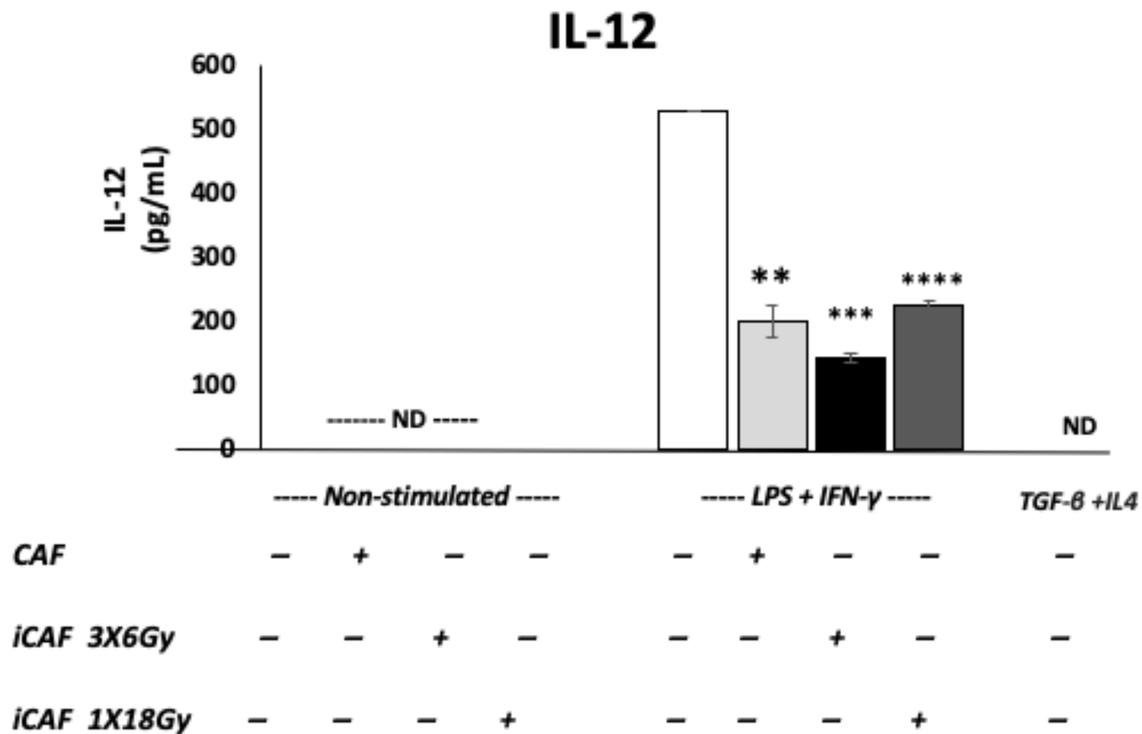


Figure. 5.36: Detection of IL-12 produced by monocyte derived macrophages co-cultured with irradiated or non-irradiated CAFs measured by ELISA assay. *Data represent the mean ±SD values from four independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF. (****p < 0.00001). ND. Non-detected. iCAF=Irradiated CAFs.*

Figure 5.37 describe the effect of both irradiated and non-irradiated CAFs on the production of IL-10 by macrophages. The expression of IL-10 by uncommitted macrophages was enhanced after co-culture with all CAF groups, however results weren't significant due to large intra-group variability. On the other hand, all CAFs group significantly reduced the production of IL-10 in M1 stimulated conditions.

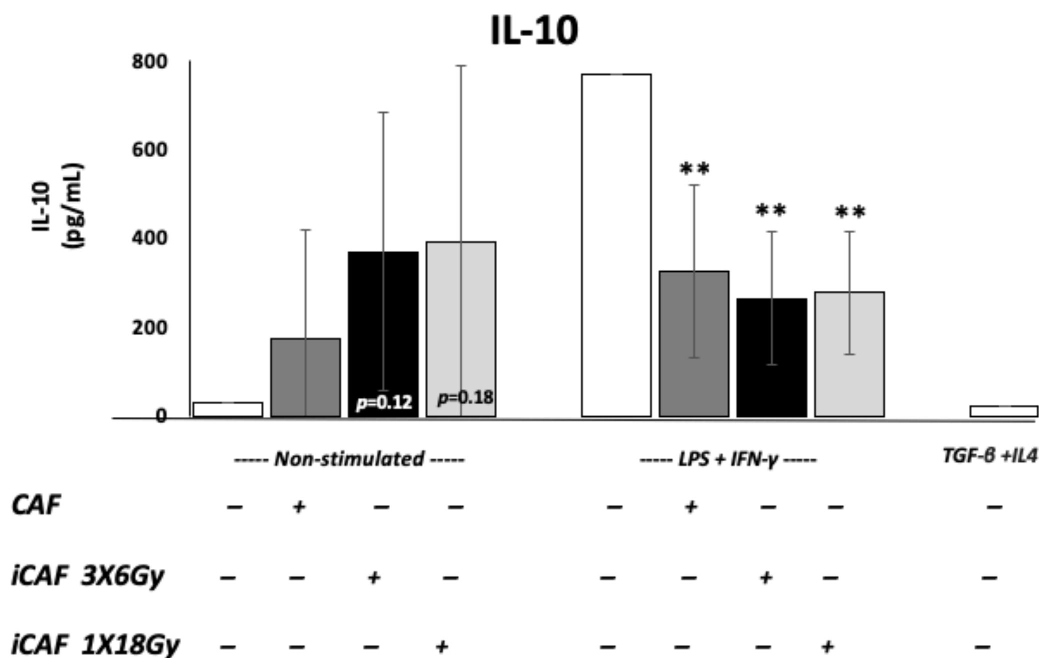


Fig. 5.37. Detection of IL-10 produced by macrophages co-cultured with irradiated or non-irradiated CAFs. *Expression of IL-10 were measured in the supernatants of both irradiated (iCAFs) and non-irradiated CAFs co-cultured with monocyte derived macrophages non-stimulated (M0), stimulated with LPS (100 ng/ml) and IFN-γ (20 ng/ml) for M1 phenotype, and stimulated with IL-4 (50ng/ml) and TGF-β (10 ng/ml) for M2-phenotype, for 48 hours by ELISA assay. Data represent the mean ±SD values from four independent experiments measured in duplicates. Student's T-test, p-value was determined between control and non-irradiated CAF, control and both irradiated CAF. (**p < 0.001). iCAF=Irradiated CAFs.*

The following tables summarize all the results gathered in our study:

Table 5.1 Cell Surface Marker Expression (M1-markers)

Macrophages	Experimental Settings	CD80	CD86	CD40
M0	*Conditioned medium*	Slight increase by all CMs (*Significant Value* for all CAF-CM)	No changes	Increase by all CMs. (*Significant Value* for irradiated CAF-CM)
	Co-culture	Slight increase by all CAF	No changes	Slight increase only by 3x6Gy group

M1	*Conditioned Medium*	Slight increase by all CM, but no significant	No changes	No changes
	Co-culture	Slight decrease by all groups	No changes	Significant inhibition by all groups, specially 3x6Gy

Table 5.2 Cell Surface Marker Expression (M2-markers)

Macrophages	**Experimental Settings**	**CD206**	**CD163**
M0	*Conditioned medium*	Strong induction by all CAF-CM (significant)	Near significant induction by all CAF-CM
	Co-culture	Induction by all groups but not significant	Slight induction by ctr CAF and 1x18Gy (non significant)
M1	*Conditioned Medium*	No changes (slight decrease by iCAF-CM)	No changes
	Co-culture	Non-significant induction by all groups	No changes. Non significant enhancement by 3x6Gy

Table 5.3 Nitric oxide production

Macrophages	**Experimental Settings**	**Nitric Oxide (NO)**
M0	*Conditioned medium*	No changes (slight significant induction by 1x18Gy)
	Co-culture	Strong significant induction by all groups
M1	*Conditioned Medium*	Significant inhibition by all CAF-CM

	Co-culture	Significant inhibition by all groups

Table 5.4 Cytokine Expression

Macrophages	**Experimental Settings**	**IL-6**	**IL-12**	**TNF-a**	**IL-10**
M0	*Conditioned medium*	Strong induction by all CAF-CM. Significant for 3x6Gy group	No changes	No changes	Strong induction by all CAF-CM
	Co-culture	Strong induction by all groups	No changes	No changes	Strong induction by all groups but not significant
M1	*Conditioned Medium*	Strong induction by all CAF-CM. Significant for ctr CAF group	Very strong inhibition by all CAF-CM	Strong inhibition by all CAF-CM	Slight inhibition over high expression values
	Co-culture	Strong induction by all groups	Very strong inhibition by all groups	Strong inhibition by all groups, significant by the irradiated groups	Strong inhibition by all groups

6 Discussion

CAFs have been portrayed as an immunomodulatory cell to create a pro-tumour inflammatory environment [53]. Macrophages recognised as versatile cells with the capacity to change their physiology according to environmental stimuli [93]. Even though several approaches have been made to understand the interaction between these two a tumour stromal cells, the entire scenario still not clear. In the present study, we have investigated the immunoregulatory effects of CAFs affecting macrophage polarisation *in vitro* and the effect of ionizing radiation on CAFs immunoregulatory capabilities. The overall main outcomes of the present study are as follows:

- CAFs, both in co-culture and by conditioned medium, promote changes on uncommitted macrophages (M0) that harmonize with both M1 and M2 phenotypes.
- CAFs, both in co-culture and by conditioned medium, abrogate some of the pro-inflammatory features of M1 macrophages as demonstrated by strong inhibition of nitric oxide production, strong inhibition of proinflammatory cytokines and a reduction of some M1 surface markers.
- Neither high dose radiotherapy (1x18 Gy) nor lower dose fractioned radiation (3x6 Gy) is affecting in general terms the immunoregulatory features exerted by CAFs over macrophages *in vitro*.

In the present study, CAFs were collected from non-small-cell-lung-carcinoma (NSCLC) tissue by enzymatic digestion and outgrowth, as described by *Gorchs et al* [155]. Fibroblasts established as primary cell cultures, unlike immortalized cell lines, have a finite proliferative potential and closely mimic *in vivo* fibroblast features. In this way, the translation of *in vitro* data to *in vivo* effects in human can be done more precisely. On the contrary, fibroblasts established cell lines show altered phenotype with frequent genetic alterations, loss of *p*53 function, directing to infinite proliferative potential [158]. One constraint that can be experienced by operating with primary cell cultures is that original *in viv*o cell features can become altered after every passage. Therefore, to assure steady results, in our study, fibroblasts were used always after the third passage or cryopreserved to use in later experiments.

Cell surface molecules are used as markers of cellular differentiation and function, imparting information into the phenotypic diversity, functional capability and activation status of the cells [159]. In our study, we used cell surface markers to categorise the polarisation status of macrophages. Surface expression of co-regulatory receptors (CD-80, CD-86 and CD-40) were

overexpressed in LPS + IFN-γ stimulated macrophages (M1) compared to unstimulated (M0) or IL-4 + TGF-β stimulated macrophages (M2). It is well documented that LPS + IFN-γ stimulated macrophages to display the high level of these co-regulatory receptors (CD-80, CD-86, CD-40), while alternatively activated macrophages (IL-4 stimulated) lack these co-regulatory receptors [160]. Moreover, the expression of mannose receptor (CD-206) was elevated in IL-4 + TGF-β stimulated macrophages while this cytokine cocktail was not able to enhance the expression of the scavenger receptor (CD-163). One previous finding describes that IL-4 strongly increase the expression and function of the macrophage mannose receptor (CD-206) [161]. On the contrary and similar to our observations, another study report that IL-4 + GM-CSF treated monocytes downregulate the expression of CD-163 [162]. Importantly, the opposite results in the expression of CD-206 and CD-163 were observed after stimulation of M0-macrophages with the anti-inflammatory agent dexamethasone [163]. This last observation and other recent studies in the field of immunology have highlighted the complexity and the oversimplified field of macrophages polarisation [164]. In-fact, pro-inflammatory (M1) and anti-inflammatory (M2) macrophages portray two groups with overlapping cellular expressions [165]. The various stimulator of M2-polarization (IL-10, IL-4 or IL-13, glucocorticoid hormones, vitamin D3) induces different functional phenotypes in macrophages. According to their diversity, M2-macrophages are further classified into different categories based on the nature of their signal inducer-M2a (IL4 + IL1-3), M2b (IL-1receptor antagonist (IL-1Ra) + Toll-like receptor ligands), M2c (IL-10 + TGF--β) and M2d (toll-like receptor + adenosine A2A agonist) [101]. Along with these lines, the function of macrophage polarisation should be considered as a practically useful tool, a simple hypothetical framing to explain a continuum of diversified functional status.

Previous studies speculated that CAFs can promote the polarization of macrophage towards M2 phenotype [166]. In the present study was elucidated that monocyte-derived macrophages incubated with CAFs, up-regulated the expression CD-206 and CD-163 from non-stimulated-(M0), and LPS + IFN-γ stimulated suggesting that CAF promote the transformation of macrophages into pro-tumour phenotype of macrophages (M2). Previous studies have suggested that CAFs-derived IL-6 and CCL2 promote the differentiation of macrophages towards M2 phenotype [167]. One previous study validated that, the supernatant of CAFs preferably induce M2 phenotype, as CD-14mRNA level increased in human monocyte-derived macrophages [104].

On the other side, macrophages exposed to CAFs or their conditioned medium show a slightly enhanced expression of co-regulatory receptors (CD-80, CD-40) in non-stimulated condition. This

slight increase in the level of M1- markers is actually the feature of M1-polarised macrophages. In this way, our study shows that CAFs function as an immunomodulatory cell modulating the macrophages polarisation in a way that resulting macrophages possess the characteristic of both M1 and M2 phenotypes. Besides this, many studies reported that polarisation of macrophages in a different type of a tumour seems more complicated, sometimes it showed more M2-phenotype with some feature of M1-gene expression[168, 169]. As most of the study describes that macrophages portray two groups of continua of overlapping cellular expressions [101, 170]. As one study described that macrophages marker expression and cytokine production *in vitro* is subject to the maturation and activation procedure [163]. Most of the macrophage-based studies used one or two markers to identify the polarisation status of the macrophage. In this way, it is very hard to assess the extent of overlapping of these markers on individual cells. As fully polarized M1 and M2 (or alternatively activated) macrophages are the extremes of a continuum of functional states [171]. Considering the macrophage plastic nature [172], the use of cell surface markers to identify the polarisation of macrophages is a bit difficult. Cell markers alone don't completely characterise the various sub-populaces of macrophages [173]. There is a need for new nomenclature, that can relate the stimuli used for macrophage activation, must explain the macrophage nature [96]. In short, a more defined and powerful M1 vs M2 discriminating system is actually needed for better recognition and understanding of macrophage phenotype.

On contrary, in LPS+ IFN-γ stimulated macrophages (M1), the expression of CD-80 and CD-40 were down-regulated in CAF-educated cells, especially in co-culture conditions. These observations suggested that CAFs suppress the pro-inflammatory features of M1-macrophages by down-modulating the expression of co-regulatory receptors. These results clearly reflect the immunosuppressive potential of CAFs in fully polarised M1-macrophages. One investigation reported that isolated fibroblasts from lung cancer patients have been reported to manifest negative co-regulatory immune signals PDL1 and PDL2, that may have an immunosuppressive effect on T-cell activation [89]. We can extrapolate these reported observations and may speculate in co-culture condition, CAFs may suppress the expression of co-regulatory receptors on fully polarised M1-macrophage in that way. There is further need to explore it. Many previous studies demonstrate that CAFs secrete CXCL12/SDF1, macrophage colony-stimulating factor (M-CSF), IL-6 and CCL2 to stimulate the recruitment of monocyte to TME and their polarisation towards M2-phenotype[133, 174]. Importantly, the recent studies reported that collaboration of CAFs and M2-macrophages is mutual, as M2-macrophage can also influence the phenotype of fibroblasts and change their reactivity [133, 174]. Previous reports revealed that M1 macrophages expressed CD-

80 and CD-86, but a subgroup of M2-macrophages also displays CD-86 [175, 176]. Most surprisingly, CD-86 is the only receptor with unchanged expression in our study, as CD-86 co-regulatory receptors are present on both M1 and subpopulation of M2-macrophages. This observation may suggest that CAFs does not modulate/regulate the expression of CD-86 by macroahages.

Furthermore, in this study, we measured the nitric oxide (NO) production by macrophages. Nitric oxide production was high in pro-inflammatory conditions (LPS and IFN-γ stimulation). Inversely, in resolving conditions with TGF-β and IL-4, the production of NO is even lower than in non-stimulated conditions. As it is generally accepted that in M1 macrophages, nitric oxide synthase (iNOS or NOS2) catalyse the conversion of arginine to NO and citrulline [154]. While M2 macrophages catalyse this arginine into ornithine and urea via arginase-1 (Arg-1) [154]. According to previous observations, CAFs support the polarization of M2 macrophages, we hypothesized that CAFs-educated macrophages downmodulate the production of NO.

The outcome of our studies displayed that CAFs significantly suppressed the secretion of NO in LPS + IFN-γ stimulated condition, while the production of NO was enhanced in non-stimulated macrophages (M0). This significant reduction of NO production in fully polarised M1-macrophages showing that CAFs has the potential to suppress the features of M1-macrophages. On contrary, CAFs induces the expression of NO in un-committed macrophages.
Based on our observations, we already suggest that these non-stimulated macrophages (M0) population has the potential to polarised both possible ways (M1 or M2). Similarly, CAFs educated macrophages have the characteristic of both M1 and M2 phenotype [177]. As CAFs induces the expression of CD-80, CD-40 and Nitric oxide in un-stimulated macrophages, which are the characteristic of M1-macrophages. In the recent past, Augsten *et al* showed that CAFs secreted chemokine (CXCL14) expression is specifically related to the nitric oxide synthase-1 activity (NOS1) [178]. This CXCL14-induced NOS1 feature of CAFs assumes an important role in tumour macrophage recruitment and tumour growth. Augsten *et al* identified NOS1 as an oxidative-stress induced competent of CAFs [178]. Here, we may suggest that CAFs-related NOS1 may have any role in this high production of NO in non-stimulated macrophages.

After describing the morphological feature and surface marker expression, we quantified the cytokine secretion to describe polarisation results. Determination of human IL-6, TNF-α, IL-12, and IL-10 was done by specific ELISA immune assays. The analysis of NO production was broadly

supported by the pattern of cytokine production. As expected, cytokine secretion of IL-6, TNF-α, and IL-12 was high in M1-stimulated macrophages, while they remained negligible in non-stimulated (M0) and TGF-β + IL-4 stimulated macrophages (M2). Macrophage cytokine profile is the reflection of macrophage heterogeneity and plasticity. A number of cytokines have both positive and negative effect on tumour growth such as IL-6, IL-10 and TNF-α (214, 215). Macrophages are the major producer of inflammatory cytokines (IL-6, IL-12, TNF-α). The LPS+IFN-γ stimulated macrophages, produce pro-inflammatory cytokines (IL-6, IL-12, TNF-α), which initiate and maintain the inflammatory microenvironment. In the context of cancer, many studies explained the up-regulation of transcription factor NF-κB in macrophages by secretion from the necrotic tissues and high-mobility group protein 1 (HMGB1), matrix-degraded-proteins [179]. Moreover, in TME, macrophages are accumulated in the hypoxic area, where hypoxia induces a hypoxia-induced factor (HIF)-1 alpha-mediated transcriptionally programmed factor, which also activates the transcription factor NF-kB in macrophages [180]. The degraded proteolytic fragment of the extracellular membrane (ECM) such as the cryptic peptide of laminin-10, can support the activation of inflammatory cells and induces the stimulation of inflammatory gene on macrophages through the activation of Toll-like receptor family members [181]. IL-12 has been showing to induce an antitumour immune response in numerous tumour model [182]. IL-12 is produced by macrophage and dendritic cell stimulate the anti-inflammatory activities [183]. IL-12 induces IFN-γ production by T-cell and NK cell, which improves the cytotoxicity by NK, NKT cells and cytotoxic-T-cells [183].

The findings of our present study elaborated that CAFs clearly block the production of IL-12 and TNF-α in M1-polarised macrophages. These results may speculate that CAFs-secreted TGF-β, IL-6, IL-10 can suppresses the function of macrophages and dendritic cells [86]. As CAFs-derived TGF-β is one of the prominent immunosuppressive cytokines in TME that can down-modulate the expression of MHC class II molecule and of co-stimulatory molecule CD-40, CD-86- CD-80, which are important for antigen presentation [86]. TGF-β also suppress the pro-inflammatory cytokines (TNF-α, IL-12 and IFN-γ) that stimulate T-cell recruitment and function [86]. A study elaborated that CAFs from hepatic carcinoma patient secrete CXCL12/SDF1 to attract monocyte-derived precursor towards TME and convert them into MDSC (Myeloid-derived suppressor cells) via the IL-6-mediated STAT3 activation pathway [184]. In a similar way, another investigation described that pancreatic stellate cells (CAFs precursor) produced various cytokine (IL-6, VEGF, M-CSF) and chemokine (CXCL-12/SDF1, CCL2/MCP-1) to stimulate differentiation of

monocyte towards MDSCs in a STAT3 dependent manner[185]. All these investigations explained that CAFs have the potential to block the production of these pro-inflammatory cytokines.

IL-6 is another pro-inflammatory cytokine. Our results elucidated that CAFs enhanced the production of IL-6 in non-stimulated (M0) and LPS + IFN-γ stimulated macrophages (M1). This observation validates that CAFs as an immunoregulatory cell, that can regulate the expression of cytokines. IL-6 is a pro-inflammatory cytokine, but CAFs stimulate the production of IL-6 in both populations. In addition to previously mentioned observations, (slight induction of CD-80, CD-40, induction of Nitric oxide), we can say that CAFs can promote the polarisation of non-stimulated macrophages towards M1-phenotype. When we compare our results with other, it showed that IL-6 has a dual role in TME. Previously, the expression of IL-6 was believed to induce monocyte activation towards M1 phenotype [186, 187]. On contrary, many other studies considered IL-6 as a pro-tumorigenic cytokine, which is highly expressed in malignancy [188]. Another recent study showed that CAFs secreted IL-6 increase tumour proliferation by promoting M2-macrophage recruitment and development [189]. One recent study investigated that tumour growth and invasive capability were attenuated when IL-6 was blocked in bladder cancer [190].IL-6 stimulates monocyte differentiation towards an M2 phenotype [189]. A previous study showed that co-culturing of fibroblast with bladder cancer cells (BCa) can assist in cancer cell invasion and IL-6 expression [177]. Our study also showed that CAFs upregulated the production of IL-6 in both polarised macrophages (MO and M1). These observations recommend that CAFs are capable to produce both pro-tumour and anti-tumour effect in the tumour [177].

Surprisingly in our results, IL-10 (an anti-inflammatory cytokine) was up-regulated in M1, but not in M2 and M0 macrophages. IL-10 is a known marker of M2-macrophages, but the results of our study are similar to former results in murine and human models [162, 191]. Another study showed that LPS + IFN-γ + immune complex-stimulated macrophages produce a high level of IL-10 [94]. One of the studies claimed that TNF-α stimulates the secretion of IL-10 by resting and LPS-stimulated macrophages as a counteractive mechanism to regulate the immune response [192]. Moreover, our results showed that CAFs as an immunosuppressive cell suppressed the production of IL-10 by M1-macrophages. On contrary, CAFs enhanced the production of IL-10 in non-stimulated macrophages (M0). This enhancement of IL-10, with the addition to the previous finding of the present study (Induction of CD-206 and CD163), we speculate that CAFs can assist the polarisation of uncommitted macrophage towrads M2 phenotype.

These ambiguous results showed the higher plastic nature of macrophages, which can sense their microenvironment and modify their polarity [193]. Meanwhile, the present study clearly mentioned that CAFs promote bothM1 and M2 features from M0-macrophages. CAFs induce expression of CD-206, CD-163 and also IL-10 which are features of M2-macrophages. While, CAFs also induce the expression of CD-80, CD-40, IL-6 and nitric oxide (NO) which are features of M1-macrophages.

Radiation therapy has a profound effect on the tumour microenvironment. It can alter the tumour cell and their related stroma including CAFs [133]. In our study, we planned to analyse the effect of ionizing radiation on CAFs-mediated macrophage polarisation. Of importance, we compared the effects of fractioned radiation vs. single high dose radiation on the polarisation of macrophages. In co-culture settings, the CAFs treated with fractioned radiation, showed an enhanced expression of both CD-206 and CD-163 in LPS+ IFN-γ stimulated macrophages. However, differences with controls were not statistically significant due to prominent inter-donor heterogeneity. A previous study stated that a low dose of fractioned radiation produced a more activated fibroblast as compared to the higher dose [194]. In this way, the CAFs subjected to low dose fractioned radiation transform the M1-polarised macrophages towards M2 phenotype by enhancing the CD-206 and CD-163 expression. In an *in vitro* model of mammary stroma, high dose radiation per fraction was more successful in decreasing the pro-tumorigenic microenvironment through prevention of fibroblasts differentiation [194].

Tumour immunity is interconnected by various soluble factors from immune cells and CAFs. Both immune cells and CAFs can have a tumour suppressing and promoting effects. CAFs produce various kind of soluble factors that play in a paracrine way to the tumour cell and their neighbour cells. CAFs generated various immunosuppressive molecules such as TGF-β1 [195], PGE2, IDO [196], CXCL-12/SDF1 [184], CCL2/MCP-1 or tenascin-C [185]. All of these molecules are involved in the immunosuppressive action of CAFs. One of the studies illustrated that CAFs supernatant contained IL-4, PGE2 and TGF-β cytokines; but the production of these cytokines not varied after exposure to a radiation dose of 18-Gy [155]. In the present study, all our results displayed that CAFs-mediated effect on the macrophage polarisation are identical for both irradiated and non-irradiated CAFs, and unrelated with the fractionation procedure and radiation dose. Previous investigations have mentioned CAFs as a radio-resistant cell which can sustain a high dose of radiation above 50 Gy [148, 149]. Despite that, CAFs exposed to a single high dose of over 10 Gy of radiation develop a senescent phenotype due to irreversible DNA damage [147].

These senescent fibroblast does not multiply, but they still produce various soluble factors to reshape the tumour microenvironment [150, 197]. In a similar way, Hellevik *et al* showed a High dose of radiation can increase the MMP3 and decrease the MMP1 expression, but it had no effect on the manifestation of other major MMPs [147]. In this way, high dose of the radiation applied the favourable inhibitory effect on the proliferative, migratory and the invasive ability of lung CAFs [147]. The present study manifested that CAF-mediated effects over macrophages are induced by paracrine signals because similar results were observed via a conditioned medium as well co-culture.

7 Conclusions and future directions

In this project, we demonstrate the presence of a cellular cross-talk between human freshly isolated CAFs from non-small-cell-lung-carcinoma (NSCLC) tissue and human freshly isolated macrophages from peripheral blood of healthy donors.

Few primary observations can be featured from this study;

- CAFs both in co-culture and by conditioned medium, promote changes on un-committed macrophages (M0) that harmonize with both M1 and M2 phenotypes.
- CAFs, both in co-culture and by conditioned medium, abrogate some of the pro-inflammatory features of M1 macrophages as demonstrated by strong inhibition of nitric oxide production, strong inhibition of proinflammatory cytokines and a reduction of some M1 surface markers.
- Neither high dose radiotherapy (1x18 Gy) nor low dose fractioned radiation (3x6 Gy) is affecting in general terms the immunoregulatory features exerted by CAFs over macrophages *in vitro*.

The immune system plays crucial roles in cancer development. On one side, tumour modifies itself at the molecular, metabolic and genetic level to gain resistant against apoptosis. On the other side, tumour cells evade the immune system by adjusting their immunogenic profile and expanding the immunomodulatory cells and immune-suppressive mediators. Plenty of work has been carried out for the understanding of the molecular pathway in the cancer cell, followed by the development of therapeutic strategies to cure cancer. However, researchers are confronted with tremendous challenges in this attempt such as tumour cells genomic instability, that is responsible for the ultimate attainment of a drug-resistant phenotype. The better understanding of the interaction

between tumour cells and stromal cells and their contribution for tumour initiation and proliferation may give us new opportunities to invent novel treatments, with the objective of targeting the genetically stable stromal cells or their paracrine factors, and in this manner prevail the drug resistance and limited the tumour development.

Nowadays, the role of CAFs in tumour formation and proliferation via its chemokine and cytokine secretions are generally accepted. Therefore, in the last few years, CAFs have been chosen as a potential therapeutic target in tumour-immune therapy. Hence, in order to develop more useful and practical therapeutic strategy against CAFs or CAFs paracrine secretions, advanced translational research on CAFs-tumour cells and CAFs-tumour stromal cells interaction are required.

It is important to indicate that CAFs incorporates the heterogeneous population of cells and the extent of heterogeneity in CAFs subtype can differ among the tumour type, tumour stage and tumour position in the body [198]. Along these lines, there are over 100 types of a tumour containing different CAFs subtypes and CAFs-paracrine factors, that may fluctuate considerably among tumour type. Furthermore, other features like the settlement of hypoxic condition in rapidly proliferating tumours may increase the production of cytokines and chemokine which is not present in high vascularized tumours. Meanwhile, our current study addressed the immunoregulatory potential of CAFs and their effect on macrophages polarisation.
In the future prospect, we can extend our study by working in various directions:

- Further study about CAFs potential for macrophage repolarization
- Continue to investigate CAFs-derived soluble factors accountable for immunosuppressive effects.
- Comparison of the secretory profile of normal lung-fibroblast isolated from non-cancerous tissue vs NSCLC-Associated fibroblast.
- Study the effect of CAFs on other cell types of immune system such as neutrophils, Natural killer cells, dendritic cells, T-reg cells.
- Study the potential connection of CAFs with infiltration of immune cells (Macrophages, NK-cells, MDSc, T-reg cells, Th2 and Th17) in lung tissue of NSCLC mice/patient.

Study the immunoregulatory potential of CAFs in animal models.

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