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Modulation of dendritic cells phenotype and functions by irradiated and non-irradiated lung cancer fibroblasts. An *in vitro* study.

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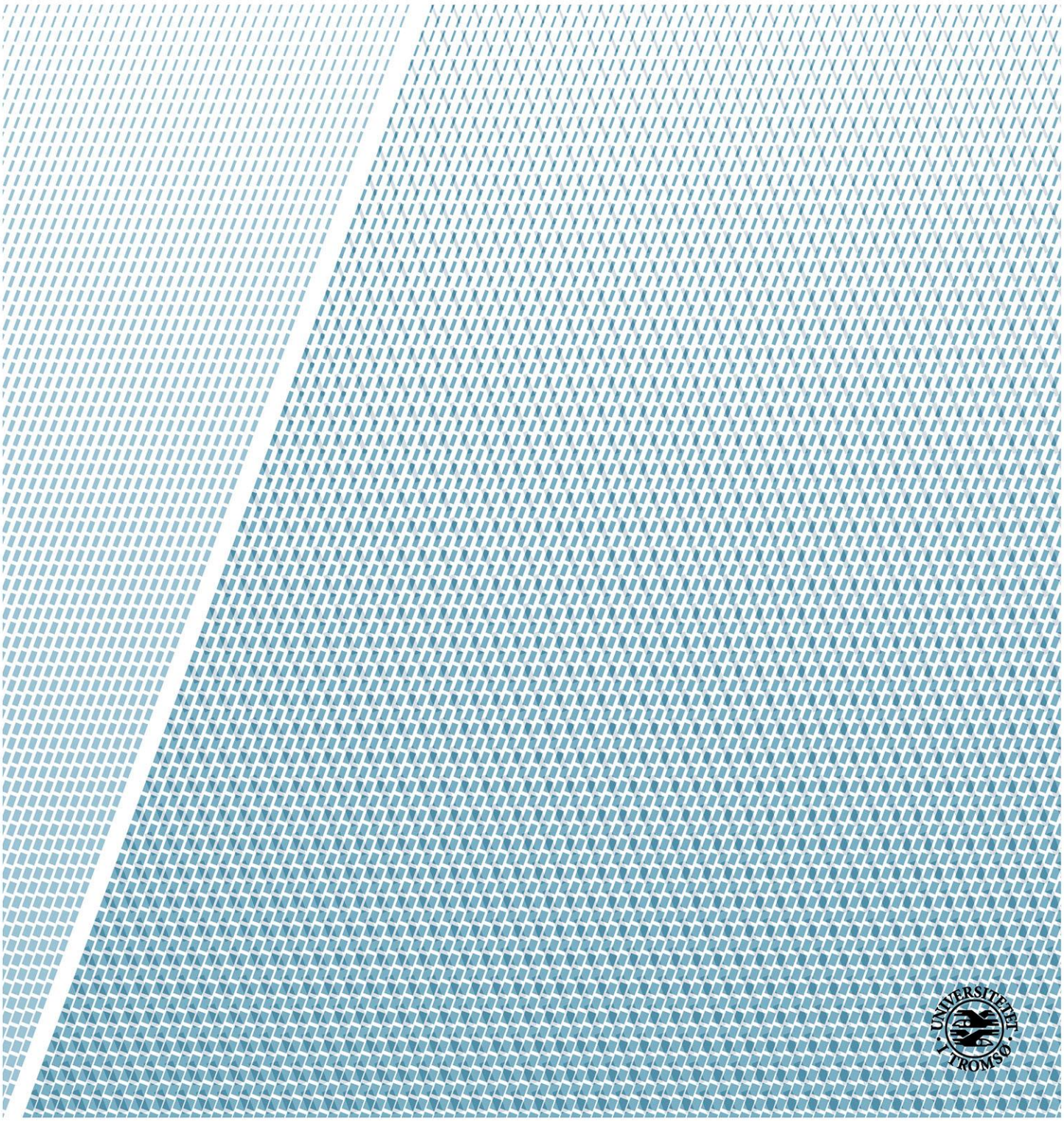
Master thesis in Biomedicine, May 2019



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Acknowledgements

I present in this thesis the work I have been part of since January 2018 at the Molecular Inflammation Research group (MIRG), Institute of Medical Biology at the University of Tromsø, Norway. This project was under the supervision of Inigo Martinez-Zubiaurre.

I want first of all to thank my supervisor, professor Inigo Martinez-Zubiaurre, for giving me the opportunity to be a part of his research group and writing my master thesis about cancer and immunology, which has been two of the most interesting subjects to me during my time at the university of Tromsø. Inigo has deep knowledge about the biology of cancer and has been able to give me insight to this complicated subject. His wife, Dr. Turid Hellevik has advised me about how to write the thesis and given me support about how to do the process easier. Both of you have been a tremendous help for me.

Many thanks to professor Tor B. Stuge for given me insight to the immunological aspects of the subjects. He has always been available to me and has helped me to better understand the correlation between tumor biology and immunology.

A special thanks goes to Rodrigo Berzaghi. He has trained me in all of the experimental methods that I have learned and used during my time at MIRG.

One major part of the study was the use of irradiated cancer-associated fibroblasts. This was done at the department of irradiation therapy, University Hospital of Tromsø. Here, Brede Pedersen and his colleagues gave us help whenever we needed to irradiate cells and I appreciate the helpful hand they have been given us during the study.

Summary

Background/aim of the study: Cancer-associated fibroblasts (CAFs) are a heterogenous population of cells found in the stroma of tumor. It is well known that CAFs are involved in many aspects of the development, progression and metastasis of cancer. The main objective in this thesis is to study how CAFs from non-small cell lung carcinoma (NSCLC) are able to regulate the immune functions of monocyte-derived dendritic cells (DCs) and to investigate if ionizing radiation have measurable effects on CAF-mediated modulation on DCs.

Methods: Primary cultures of CAFs were isolated from newly resected NSCLC tumor specimens (n=4) gathered at the Tromsø University Hospital, whereas buffy coats from unrelated healthy donors were used for isolation of peripheral blood mononuclear cells (PBMCs). $CD14^+$ monocytes were isolated from PBMCs and stimulated to generate immature monocyte-derived dendritic cells (iDCs) and mature monocyte-derived dendritic cells (mDCs). The experimental settings comprised both DC/CAFs co-cultures and incubation of DCs with CAF-conditioned medium (CAF-CM). Effects from either non-irradiated or irradiated (3x6 Gy or 1x18 Gy) CAFs were compared. DCs phenotype and function were determined by; expression of cell surface activation markers (CD14, CD1a, CD209, CD40, CD80, CD86 and HLA-DR), functional assays (antigen uptake, DCs migration T cell priming) and cytokine production (IL-10 and IL-12).

Results: We demonstrated that CAFs in both CAF-CM and co-culture experiments have the capability to suppress functional markers for DCs. In addition, CAFs inhibited DCs ability to internalize antigens, migratory capacity, priming of T cells and down-regulated production of IL-10 and IL-12 by DCs. Further, irradiation of CAFs, especially with fractioned regiments curtailed the inhibitory effects exerted by CAFs over DCs.

Conclusion: This study demonstrated that CAF-derived soluble molecules could mediate immunosuppressive effects upon DCs. Further, results illustrate that irradiation of CAFs with fractioned medium-high dose revert the immunomodulating function of CAFs.

Abbreviations

CAFs: Cancer-associated Fibroblasts

NSCLC: Non-small cell lung carcinoma

PBMCs: Peripheral blood mononuclear cells

iDCs: Immature monocyte-derived dendritic cells

mDCs: Mature monocyte-derived dendritic cells

CAF-CM: CAF-conditioned medium

PD-L1: Programmed death-ligand 1

NK: Natural killer cell

MDSCs: Myeloid-derived suppressor cells

MHC: Major histocompatibility complex

TME: Tumor microenvironment

ECM: Extracellular matrix

ATP: Adenosine triphosphate

OXPHOS: Oxidative phosphorylation

TGF-β: Transforming growth factor β

iNOS: Inducible nitric oxide synthase

IDO: Indoleamine-2.3-dioxygenase

IL: Interleukin

Treg: Regulatory T cell

TAMs: Tumor-associated macrophages

Th17: T helper 17 cell

DCreg: Regulatory dendritic cell

TI: Tumor-infiltrating

TCR: T cell receptor

ROS: Reactive oxygen species

PDGF: Platelet-derived growth factor

bFGF: Basic fibroblast growth factor

α-SMA: α-smooth muscle action

FAP: Fibroblast activation protein

FSP-1: Fibroblast-specific protein-1

VEGF: Vascular endothelial growth factor

HIF-1: Hypoxia inducible factor-1

NKG2D: Natural killer group 2D

APC: Antigen presenting cell

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

SDF-1**:** Stromal derived factor-1

MMPs: Matrix metalloproteases

ETM: Epithelial-mesenchymal transition

HGF: Hepatocyte growth factor

PGE_2: Prostaglandin E2

TNF: Tumor necrosis factor

NO: Nitric oxide

M-CSF: Macrophage colony stimulating factor

GM-CSF: Granulocyte-macrophage colony stimulating factor

ER: Endoplasmic reticulum

PPRs: Pattern recognition receptors

TLRs: Toll-like receptors

DAMPs: Damage-associate molecular patterns

NF-κB: Nuclear factor κB

CCR7: Chemokine receptor 7

HMGB1: High-mobility group box 1 protein

DcR3: Decoy receptor 3

DCreg: Regulatory dendritic cell

RT: Radiotherapy

DMEM: Dulbecco's modified medium

FBS: Fetal bovine serum

PBS: Phosphate buffered saline

DMSO: Dimethyl sulfoxide

CM: Conditioned medium

MV: Megavoltage

MACS: Magnetic activated sorting separation

MFI: Mean fluorescence intensity

TolDC: Tolerogenic dendritic cells

1 Introduction

1.1 Tumor immunology: General Principles

Immunology is a branch within biology that studies the immune system of different organisms. The immune system works to eradicate foreign pathogenic microorganisms and other materials that are regarded as a threat to the body and is generally divided in two different branches; **the innate and the adaptive immune system** [1]. The innate immune system can be regarded as the first line of defense and is comprised of both soluble recognition molecules as well as cellular components. Cells in the innate immune system includes phagocytic cells (e.g. **macrophages**), antigen presenting cells (e.g. **dendritic cells**) and killer cells (e.g. **natural killer cells**). These cells preform a non-specific elimination of a foreign microbial invasion or tissue damage and are very effective [2]. In comparison, the adaptive immune system has a specific reaction to a unique danger signal. The adaptive immune system consists of antigen specific T- and B cells and also a broad diversity of antibodies and cytokines produced by the cellular components of the system [3].

An immune response is orchestrated in two phases; the activation/effector phase and the resolution/immunosuppressive phase. Tumor immunology investigate the correlation between the resolution/immunosuppressive phase and cancer, since tumors try to disrupt signals in this phase to avoid immune responses [4]. Cancer cells form when non-reversible changes occur in the DNA of cells, which allows these cancer cells to lose normal cellular regulatory processes [5]. The immune system will detect these cancer cells as a threat and launch an attack to eliminate the uncontrolled cells in a process known as immunosurveillance [6]. The conception that there is a link between the immune system and cancer was proposed by Rudolph Virchow over 150 years ago and this concept has now been acknowledged for over a century [7]. But it was Paul Ehrlich who first hypothesized that cancers can be eliminated by the immune system [8]. Burnet and Thomas later supported the hypothesis of Ehrlich which lead to the theory of immunosurveillance [6].

1.1.1 Cancer immunoediting: The 3 “Es” model

Recent years of research have led to an extended concept of the immunosurveillance theory. This new conception is called cancer immunoediting and tries to explain the different phases that can occur in relation to immune system – cancer interactions. The process of immunoediting is a dynamic process and is composed of three different phases; **elimination, equilibrium and escape** (**Figure 1.1)** [9].

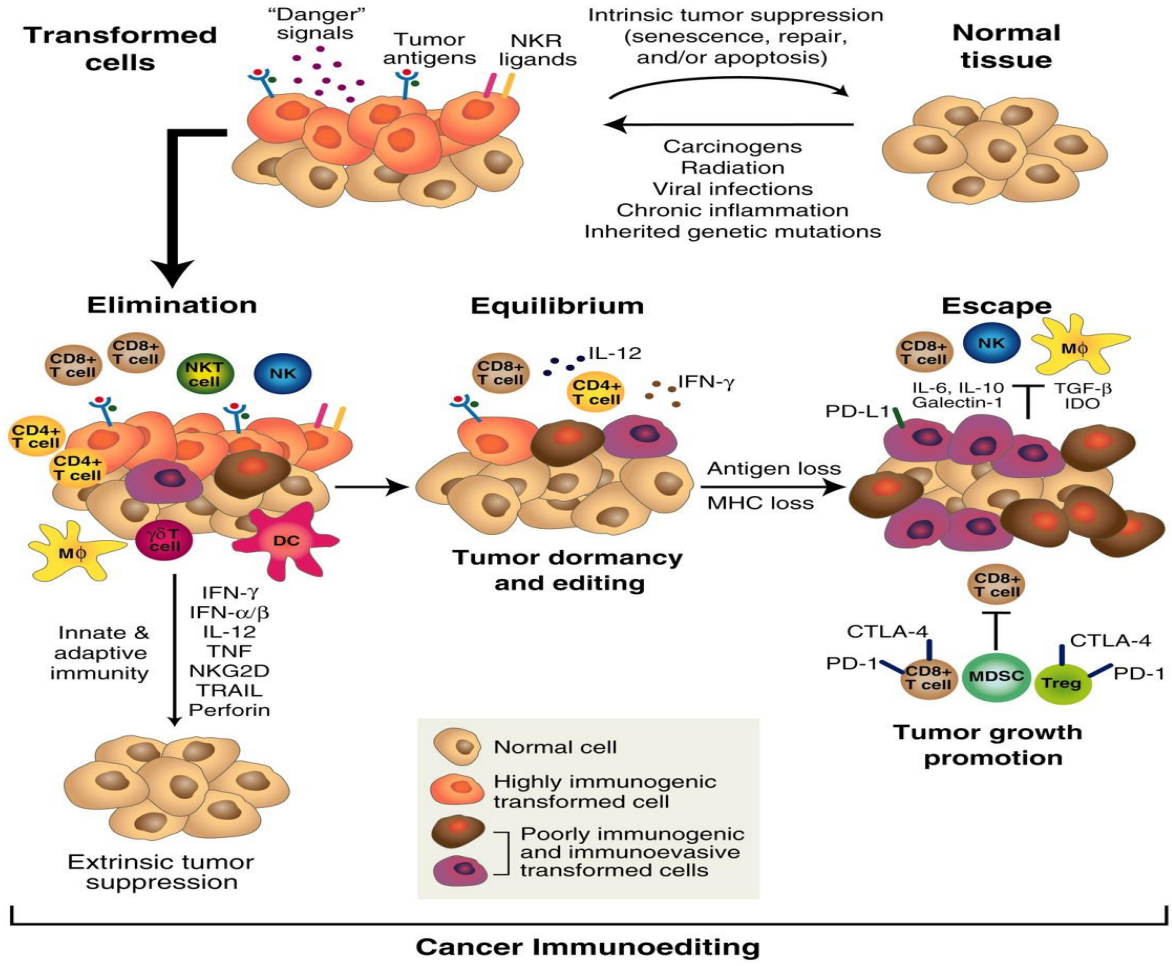

Figure 1.1: The three "Es" of cancer immunoediting: *Normal cells are able to break free from control mechanisms due to changes in DNA and generates cells with corrupt DNA. The first "E" describes the phase of elimination were immune cells are able to recognize and eliminate cancer cells before the tumor becomes clinically detectable. If some variant of the cancer cells is not eliminated, these cells may introduce the second phase of immunoediting and the next "E", which stands for equilibrium. In this phase, T cells and other factors keep the tumor in a dormant state and might be able to stop the tumor from growing further. The final "E" stands for escape and represents the last phase of immunoediting. Tumor cells can adapt genomic changes that allow them to escape recognition by the immune system and continue growing to a possible clinically detectable tumor [10].*

The first phase of cancer immunoediting is **elimination** and refers to immunosurveillance. In this phase, both the innate and the adaptive immune system works together in recognition and elimination of normal cells transformed to cancer cells that have gained the ability to escape tumor suppression through blocking of intrinsic cell mechanisms. In this elimination process, which can be thought of as a selection process, some subclones of tumors can circumvent the elimination process due to the genetically instable nature of tumor cells that could lead to downregulation of antigen presenting molecules and/or an increase in programmed death-ligand 1 (PD-L1) expression enabling tumors to not be eliminated by the immune system [11].

Equilibrium is the second phase of cancer immunoediting were some tumor cells have escaped the elimination phase. In this stage of the cancer immunoediting concept a dynamic balance take place between cells of the immune system and tumor cells. Some studies have found that high numbers of $CD8^+$ T cells, natural killer (NK) cells, γδT cells and low numbers of NKT cells, $Foxp3^+$ Treg cells and myeloid-derived suppressor cells (MDSCs) can be correlated with control of tumor growth and keep the tumor in the equilibrium phase [12].

The third "E" and final phase is **escape**. There are several factors that can lead to immune escape for tumors, including loss of recognition by the immune system, reduced expression of major histocompatibility complex (MHC) class I molecules and/or co-stimulatory molecules. Regardless why, the result is that the immune system fails in restriction of tumor growth and tumor cells will be capable to grow into a clinical detectable tumor [9].

1.2 Biology of tumor microenvironment

Tumors are complex tissues formed by malignant cells and a combination of non-malignant components that together gives support to the tumor and is frequently referred to as tumor microenvironment (TME) or tumor stroma. The TME consist of many different cellular and acellular components such as extracellular matrix (ECM), illustrated in **Figure 1.2**. Other cells besides the tumor cells includes mesenchymal cells, immune cells and vascular cells. These cells together with non-cellular factors of the ECM contribute to the heterogeneity of tumors in numerous ways. One main difference between tumor cells and other cells found in the TME is that tumor cells have been formed by several mutations in DNA that has led to the formation of cells able to grow uncontrolled. Non-malignant cells found in the TME are cells with intact DNA, but are corrupted by the tumor cells to contribute to the progression of cancer [13].

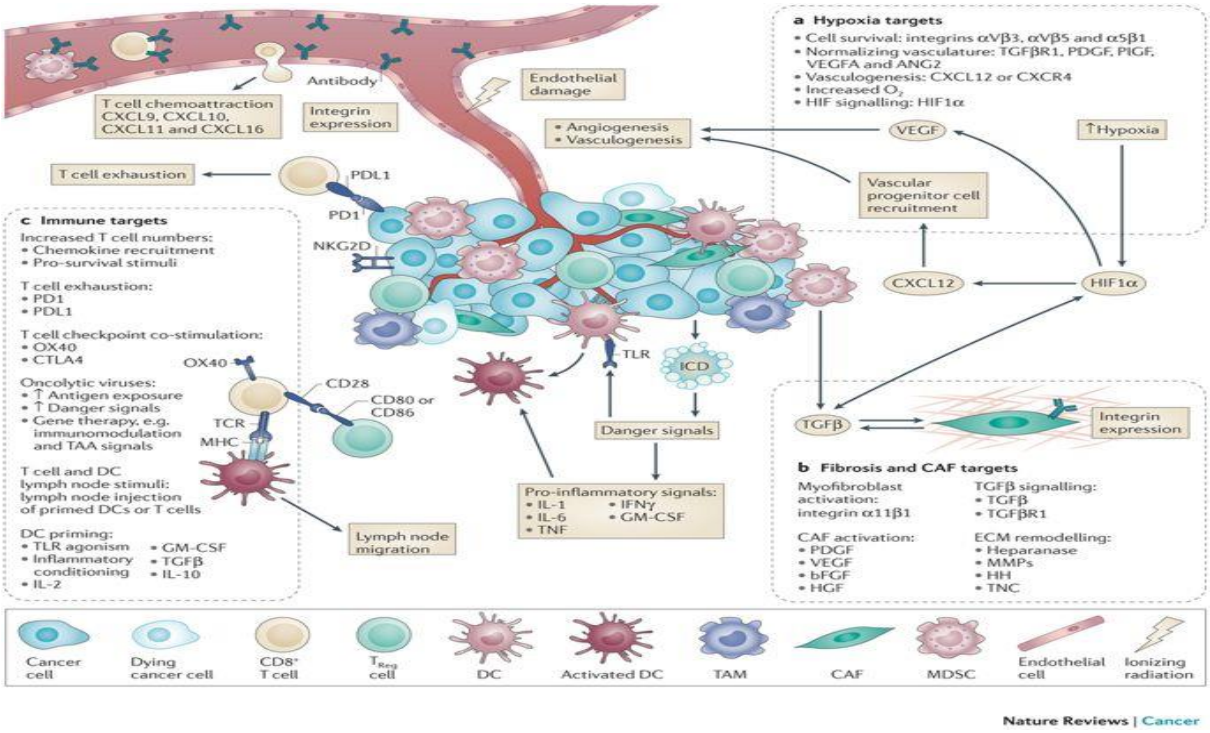

Figure 1.2: Overview of the tumor microenvironment: *TME consists of a broad diversity of cells and ECM that contributes to the heterogeneity of tumor stroma. Cells found in tumor stroma includes fibroblasts, vascular endothelial cells and immune cells [14].*

1.3 Mechanisms of cancer immune evasion

If the immune system should fail, cancer cells evade detection and eradication by the immune cells and can grow uncontrolled. Several different mechanisms, or a combination of them, can be used by cancer cells for being able to break free from the control of the immune system.

1.3.1 Antigenicity

The immune system distinguish normal cells from malignant cells by antigens presented on the surface of cells [15]. These "foreign" antigens or neo-antigens produced by cancer cell emanate from genetic mutations in tumor cells, which is a hallmark of cancer, and is one of the primary factors for generating specific neo-antigens [16]. The process were the immune system recognize and kills cancer cells can be referred to as the Cancer-Immunity Cycle, which is a stepwise process. In general, immune cells recognize the tumor antigens and generates an immune response to eliminate cells with the specific "foreign" antigens displayed on their surface [17]. Still, the tumor cells can acquire the ability to hide their identity as malignant cells. One of the most common strategies for cancer cells to avoid being identified by immune cells is to downregulate the expression of MHC class I molecules on their surface and by doing so also their "foreign" antigens [18, 19].

But the immune system has a method for detecting and destructing cells that downregulate their expression of MHC class I molecules. One of the cell types found in the innate immune system, NK cells, have the ability to identify and kill cells that downregulate the expression of MHC class I molecules [20]. Cells with unnaturally reduced expression of MHC class I molecules fail to provide NK cells with enough inhibitory signals to retain the NK cells in an inactivated state thus activating the NK cells. This enables the NK cells to kill target cells by lysis [21]. However, tumors can use other suppressive signals to evade immune attack. The Natural Killer Group 2D (NKG2D) receptor is expressed by NK cells and is one of the key receptors in recognizing stress-induced ligands" such as MICA, MICB and ULBP1-6. The interaction NKG2D/NKG2D ligand is one of the important mediators of recognizing and killing tumors. But tumor cells can manipulate the expression of NKG2D ligands on a post-transcriptional or post-translational level which reduces the expression of stress-signals and thus escaping immune recognition by NK cells [22].

1.3.2 Co-stimulatory and co-inhibitory receptors interactions

Cancer evasion strategies include the regulation of different co-stimulatory and co-inhibitory receptors displayed on the surface of immune cells. The full activation of T cells requires two signals. The primary signal is mediated through T cell receptor (TCR) – MHC molecule interactions. The secondary signal is provided through co-stimulatory/co-inhibitory molecules presented on the surface T cells (**Figure 1.3)**. These co-stimulatory/co-inhibitory molecules can interact with surface receptors on antigen presenting cells (APCs) [23]. The process from antigen recognition to killing of cancer cells are in the Cancer-Immunity Cycle divided in two phases; the priming phase and the effector phase [24]. Co-stimulatory molecules are necessary in the priming phase to activate T cells by antigen presenting cells (APC), such as B7-1 (CD80)/B7-2 (CD86) – CD28 interactions, which is one of the strongest signals in activating T cells. But the expression of co-stimulatory molecules for immune cells can be downregulated in tumor stroma [25], and lacking the co-stimulatory signals through B7-1/B7-2 could lead to a hyperresponsive state/anergy for T cells [23].

The co-stimulatory molecules CD80/CD86 also have affinity for cytotoxic T-lymphocyte associated protein 4 (CTLA4) on T cells. The interaction provide T cells with an inhibitory signal, limiting the priming of T cells and an immune response [26]. Another important interaction regarding immune response is between PD-1L and PD-1. Various cells express PD-1L, for example tumor cells, CAFs and inflammatory cells [27-29]. These cells can interact

with PD-1 found on T cells and the interaction can lead to suppression of T cells responses [30]. The balance between these stimulatory and inhibitory signals are important for the effect of the immune response. Newer studies have demonstrated that immune checkpoint can be expressed by tumor cells, which can provide cancer cells with properties for evading the cytotoxic capability of T cells [31].

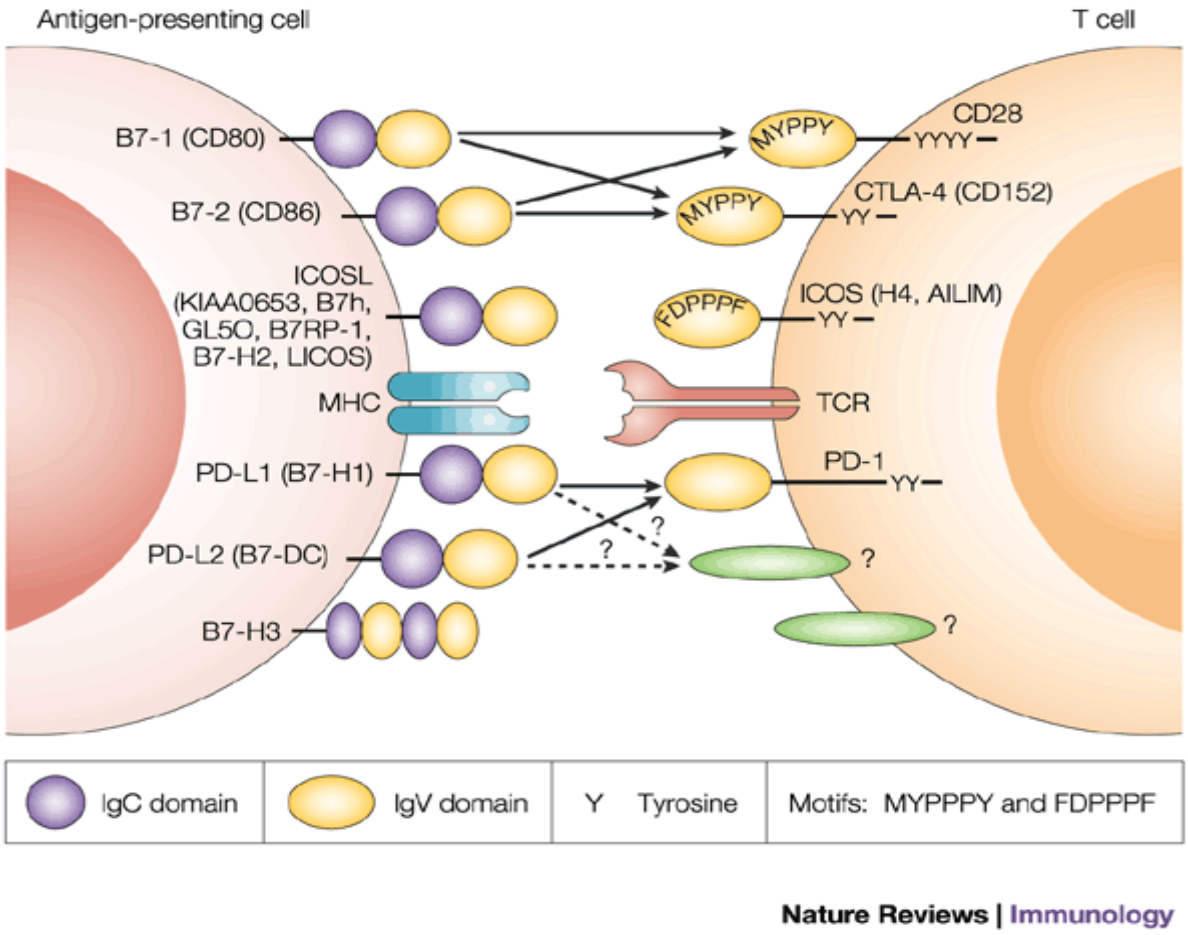

Figure 1.3: Co-stimulatory and co-inhibitory signaling: *A broad diversity of molecules are expressed on the surface of APCs and T cells. Some interactions provide co-stimulatory signals, which leads to the priming of T cells, such as B7-1/B7-2 and CD28. An inhibitory signal, on the other hand, is produced when B7-1/B7-2 interacts with CTLA4, which could result in reduced priming of T cells. Another important interaction is between PD-1 – PD-1L, which leads to suppression of T cell response [32].*

1.3.3 Avoiding cell death

Immune cells and other factors, such as stress, can induce cell death (apoptosis) in cancer cells. The process of apoptosis is a cellular suicide program that gives an organism the possibility to eliminate unwanted cells [33]. Tumor cells have the ability to resist cell death and become immortal, which is a hallmark of cancer, and lies at the heart of all tumor development [34].

Cancer cells are, in contrast to normal cells, suffering from different forms of stress, such as genomic instability, cellular hypoxia and oncogenic stress [35]. Cancer cells also experience other factors such as DNA damage and growth factor deprivation [36]. All these factors would in normal cells lead to cell death trough the intrinsic pathway of apoptosis, but cancer cells gain mechanisms to avoid this by suppressing pro-apoptotic protein expression. Important initiator molecules for the intrinsic pathway of apoptosis are BH3-only molecules, which belongs to a family of proteins called Bcl-2 family [37]. The BH3-only molecules are pro-apoptotic, but there are also other members of the same family of proteins that are anti-apoptotic, and the balance between these pro-apoptotic and anti-apoptotic molecules decides if a cell enters intracellular death program or not [38]. Most of human cancers acquire mutations in the p53 tumor suppressor gene, which shut down the expression of p53 proteins. The p53 protein regulates the process of apoptosis through interactions with Bcl-2 proteins, and when the p53 is inactivated, this leads to an elevated concentration of anti-apoptotic Bcl-2 family proteins, which tips the balance in favor for anti-apoptotic molecules which support cancer cell survival [39, 40].

1.3.4 Cellular metabolism

Cells are dependent on energy to fulfill their functions. One of the most important energy sources for cells is glucose, but cells can also use lipids or amino acids to generate energy in the form of adenosine triphosphate (ATP). The primary pathway of generating energy for cells in the presence of oxygen is through mitochondrial oxidative phosphorylation (OXPHOS), which generates more energy by oxidization of glycose in comparison to aerobic glycolysis [41]. In 1956, the German scientist Dr. Otto Warburg stated that cancer cells rewire their metabolism and start to generate energy through aerobic glycosylation even in the present of oxygen. This process is later known as the Warburg effect [42]. Aerobic glycosylation seems to be an inefficient way to generate energy for cells, since more ATP is generated from OXPHOS compared to aerobic glycosylation. But, the rate of glucose metabolism is 10-100 times faster in aerobic glycosylation than the total oxidation of glucose in mitochondria, which explain why aerobic glycosylation provides cancer cells with their high demand in energy more suitable than oxidation in mitochondria [43].

But the change in metabolism for cancer cells towards Warburg biology does not only support cancer cells with energy. It also contributes to immune suppression as well. Immune cells, like all other cells need energy to function. Cytotoxic T cells and NK cells are crucial for elimination

of cancer cells. Upon activation, the metabolic demand for these immune cells increases and there is a competition between cancer cells and immune cells for required metabolites in the TME. When cancer cells starts to increase their consumption of glucose, less becomes available for immune cells and can result in limited functions for the immune cells crucial for elimination of tumor cells [44].

1.3.5 Immunosuppressive cells

Tumors can escape the immune system by triggering the recruitment of immunosuppressive cells. Recruitment is mediated by tumor-induced cytokine production, such as transforming growth factor-β (TGF-β) and $CXCL_5$ [45]. Mechanisms used by immunosuppressive cells to dampen immune attack include; reduce antigen presentation by DCs, increased production of arginase, inducible nitric oxide synthase (iNOS) and indoleamine-2,3-dioxygenase (IDO) to prevent the proliferation and activation of B- and T cells, secretion of immunosuppressive cytokines IL-10 and TGF-β, and inhibition of the cytotoxic function of T lymphocytes and NK cells [9]. In **Figure 1.4**, an illustrated overview can be seen of immune cells recruited to the TME and signal molecules involved in the recruitment.

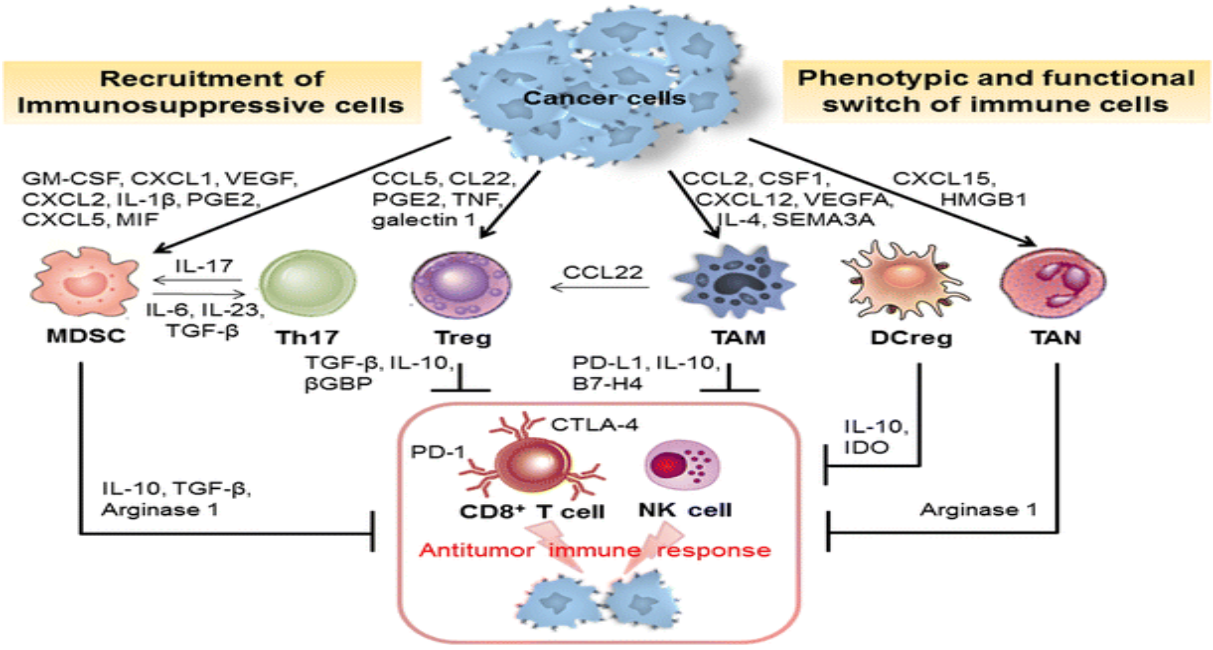

Figure 1.4: Immunosuppressive cells: *Cancer cells secrete a broad diversity of cytokines and chemokines which promotes the recruitment of immunosuppressive cells such as MDSCs, regulatory T cell (Treg), tumor-associated macrophages (TAMs), T helper 17 cell (Th17), regulatory dendritic cell (DCreg), tumor-associated neutrophil (TAN) and regulatory B cells (Breg). These cells contributes to produce a immunosuppressive environment in TME that can switch the phenotype and function of immune cells to become tumor-promoting [45]*

One of the immune cells recruited into the TME are tumor-infiltrating (TI) **Tregs**. The recruitment is mediated through mechanism involving CCL22 produced by tumor cells and TAMs [46], which is a ligand for CCR4 found on T_{reg} [47]. Accumulation of Tregs in tumor stroma can lead to modulating of function for other immune cells recruited to the tumor by various mechanism. Normally, Tregs function to maintain immune homeostasis. This is accomplished by different suppressive cellular and humoral mechanisms, which involves surface receptors CTLA-4 and CD25, and the production of inhibitory cytokines such as IL-10, TGF-β and IL-35. T_{reg} cells can also degrade ATP and express granzyme and/or perforin, which enables Tregs to kill other cells [48]. The functions for Tregs is illustrated in **Figure 1.5**. Th17 T helper cells represents a $CD4^+$ T cell lineage different from Th1, Th2 and Tregs. This inflammatory cell type is found in significant numbers inside tumors, where Th17 T cells secrete the pro-inflammatory cytokine IL-17 contributing to tumorigenesis [49].

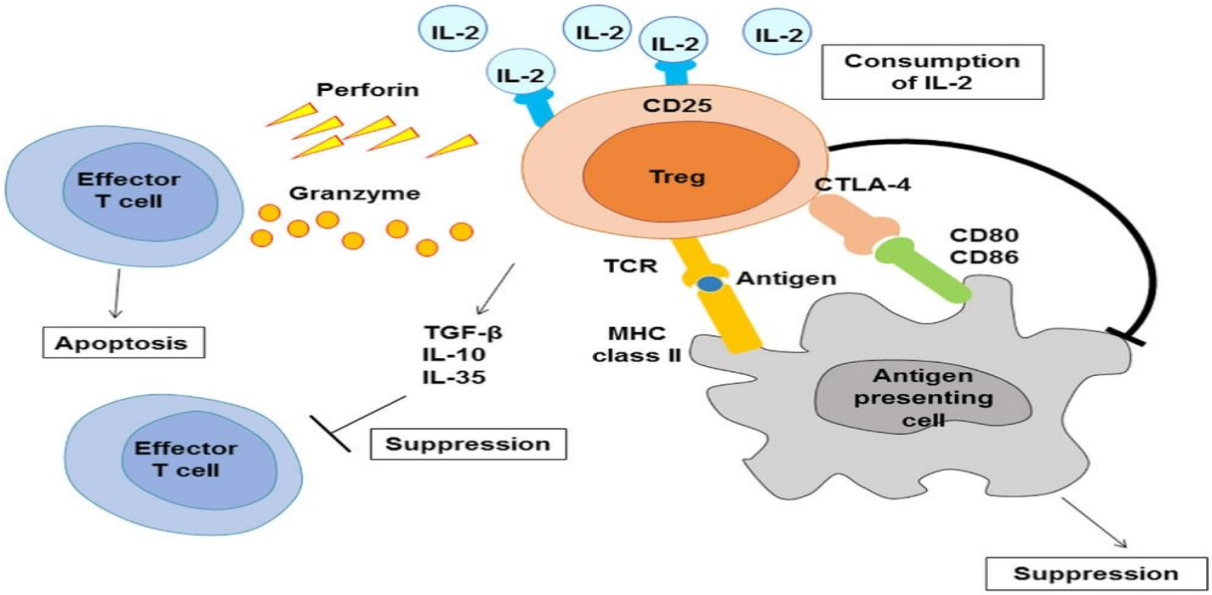

Figure 1.5: Functions of T_{reg} cells: *The suppressive functions of Tregs are mediated through the CTLA-4 pathway, expression of immunosuppressive cytokines IL-10 and TGF-β, and killing target cells through secretion of granzyme and/or perforin [48].*

MDSCs are also one of the major cell types recruited to TME and that facilitate tumor immune evasion. This type of cell represents a heterogenous population of cells which participates in numerous different immunological functions because they are able to differentiate into granulocytes, macrophages or DCs [50]. MDSCs are recruited to TME by a broad range of different chronic inflammatory factors, such as IL-1β, IL-4, IL-5, IL-10, TGF-β and many other factors [51].

Once the MDSCs are in the TME, a vast spectrum of different suppressive molecules are secreted to tumor stroma. Arginase is expressed by MDSCs and is a crucial molecule in urea cycle for converting L-arginine to L-ornithine and urea, which reduces available L-arginine for T cells. T cell uses L-arginine in metabolic pathways for survival and anti-tumoral activity [52], and the lack of L-arginine leads to translational blockage for infiltrating T cells causing inhibition of T cell proliferation [53, 54]. In addition, this results in downregulation of TCR ξ-chain, which is essential for T cell signaling and causes T cell anergy [55]. iNOS is expressed by MDSCs to catabolize L-arginine, which could lead to T cell anergy as well [56]. MDSCs can also express PD-L1 used to suppress T cells responses directly. Other molecules expressed by MDSCs are reactive oxygen species (ROS) and TGF-β, which can suppress NK cells synthesis IFN-γ [57]. There are two types of macrophages; type I (M1) and type II (M2). The M1-polarized macrophages have an inflammatory function while M2-polerized macrophages also known as TAMs, have anti-inflammatory activity [58]. TAMs are heterogenic cells due to their monocytic precursor, but also based on their various functions. These alternative activated M2-polerized macrophages are activated by IL-10 and TGF-β, and exert their function by secretion of an anti-inflammatory and wound-healing cytokine profile, which includes IL-4, IL-10 and IL-13 [59]. Another important immunosuppressive cell in the TME are stromal fibroblasts or CAFs. The biology of this cell type including their immunoregulatory functions will be described in more detail later in the introduction.

1.4 Cancer-associated fibroblasts (CAFs)

CAFs represent a major component of the tumor stroma and are involved in many aspects in the progression of tumors [60]. To understand the role of fibroblasts in cancer development, knowledge has been transferred from the concept of wound healing. Tumors are frequently seen and understood as “wounds that do not heal”. This was first identified by Rudolf Virchow in 1863 in the way that “chronic irritation and inflammatory hyperplasia are predispositions for cancer development”. This way of thinking in relation to cancer was further denoted almost 90 years later in 1974 by Alexander Haddow who proposed that “tumor production is a possible overhealing”. But the notion that tumors are wounds that did not heal was not proper recognized before Harold F. Dvorak in 1986 published an assay in the New England Journal of Medicine titled “Tumors: Wounds that do not heal” [61].

Fibroblasts are an abundant cell type found in connective tissue throughout the body. These cells become activated when tissues are damaged, and undergo differentiation into myofibroblasts [62]. Normally, the physiological role of myofibroblasts is synthesis and secretion of ECM components, mostly collagens, to influence the composition of ECM, maintaining tissue homeostasis and inflammation, and differentiation of surrounding cells. Because of these features, CAFs can be seen as architects of TME [63, 64]. Myofibroblasts are destined for apoptosis when repair of damaged tissue is finalized [65]. When cancer cells start to grow, this is interpreted as a wound for the body, which activates fibroblasts. But in this situation, the activated fibroblasts fail to undergo apoptosis and are able to support and become an critical factor of the tumor stroma [66]. Cancer cells recruit and activate CAFs by secretion of many different signal molecules within the TME, and one of the main factors are TGF-β. But other pro-fibrotic factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and IL-6 are important as well [67].

1.4.1 The heterogenic nature of CAFs

The majority of CAFs originate from resident fibroblasts [68]. But CAFs can also derive from other precursor cells such as epithelial cells [69], endothelial cells [70], resident stem cells [71], transdifferentiated smooth muscle cells [72], trans-differentiated adipocytes [73], bone-marrow derived mesenchymal cells [74] and local mesenchymal cells [75] **(Figure 1.6).** CAFs are able to maintain their phenotype for several passages when cultured *in vivo* compared to normal fibroblasts. This suggest that CAFs have been through genetic or epigenetic changes which results in the special characteristics of CAFs [76, 77].

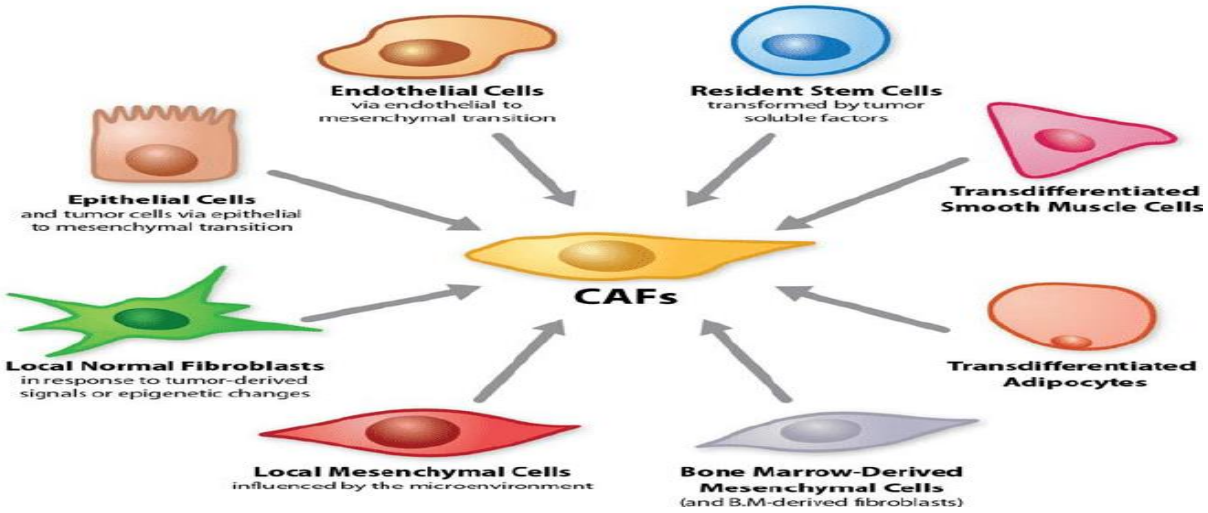

Figure 1.6: Origin of cancer-associated fibroblasts: *CAFs can originate from various populations of cells by different mechanism. This includes endothelial cells, resident stem cells, transdifferentiated smooth muscle cells, transdifferentiated adipocytes, bone marrow-derived mesenchymal cells, local normal fibroblasts and epithelial cells [78]*

There is variation between CAFs due to the heterogenic nature and high plasticity of CAFs, which makes it hard to determine specific markers for CAFs. But there has been established certain positive markers to identify and isolate CAFs from other cells [79]. The most used positive marker for identification of CAFs are α-smooth muscle actin (α-SMA) even though not all functionally activated CAFs express this marker [80]. Other markers used for CAFs are fibroblast activation protein (FAP) [81], tenascin-c (TNC) [82], podoplanin (PDPN) [83] and neuron-glial Antigen-2 (NG2) [84]. CAFs also increased the secretion of ECM proteins such as fibronectin and type I collagen, which can also be used as functional markers [85]. Cell-associated molecules, such as platelet-derived growth factor receptors a/b (PDGFR a/b), vimentin (VIM), fibroblast-specific protein-1 (FSP-1) and periostin (POSTN) are markers for fibroblast as well, but not specific markers for CAFs. **Table 1.1** represents a list of positive surface markers for both normal fibroblast and CAF [79].

Table 1.1: Common markers for identification of normal fibroblasts and CAFs

Positive markers	Comments	Surface marker
CAF markers:		
FAP	Mainly expressed by non-myofibroblast subpopulations of CAFs	Yes
αSMA/ACTA2	Consider to be the most positive marker for CAFs	No
MFAP5	The expression of MFAP5 varies amongst the populations of CAFs	No
COL11A1	Suggested to be very specific for identification of CAFs	No
TN-C	A myofibroblast-associated marker. Important factor for metastasis	No
PDPN	Can be overexpressed by some subclasses of CAFs. Also expressed by tumor cells and macrophages	Yes
ITGA11	Upregulated by CAFs related to non-small cell lung cancer	Yes
NG2	Marker of some CAF subclasses. Numerous other cells also express this marker such as myeloid and T cells	Yes
Fibroblast markers		
PDGFRα/β	Very common marker for identification of fibroblasts	Yes
VIM	A widely expressed marker by all fibroblasts	No
FSP-1S100A4	Common used marker for quiescent fibroblasts	No
POSTN	Expressed by both normal fibroblasts and CAFs	No
Col1	Not exclusive expressed by fibroblasts	No

Newer studies have suggested that different subclasses of CAFs might co-exist within tumor stroma. An illustration of the hypothesis can be seen in **Figure 1.7**. It is proposed that one subclass of CAFs are restraining tumor development (F1), while another subclass can support tumor (F2). There is also hypothesized that some specialized CAFs has a secretory phenotype, which can support tumor immunity, cancer cell survival, apoptosis and angiogenesis (F3) and some CAFs with a particular phenotype enabling them to remodel ECM through secretion of ECM components (F4) [86]. This has been confirmed by newer studies. In 2018, Bartoschek et al were able to identify three different subpopulations of breast-cancer related CAFs by techniques using a negative selection strategy in combination with single cell RNA sequencing for detection of different types of CAFs based on their transcription of genes and thereby their phenotype [87].

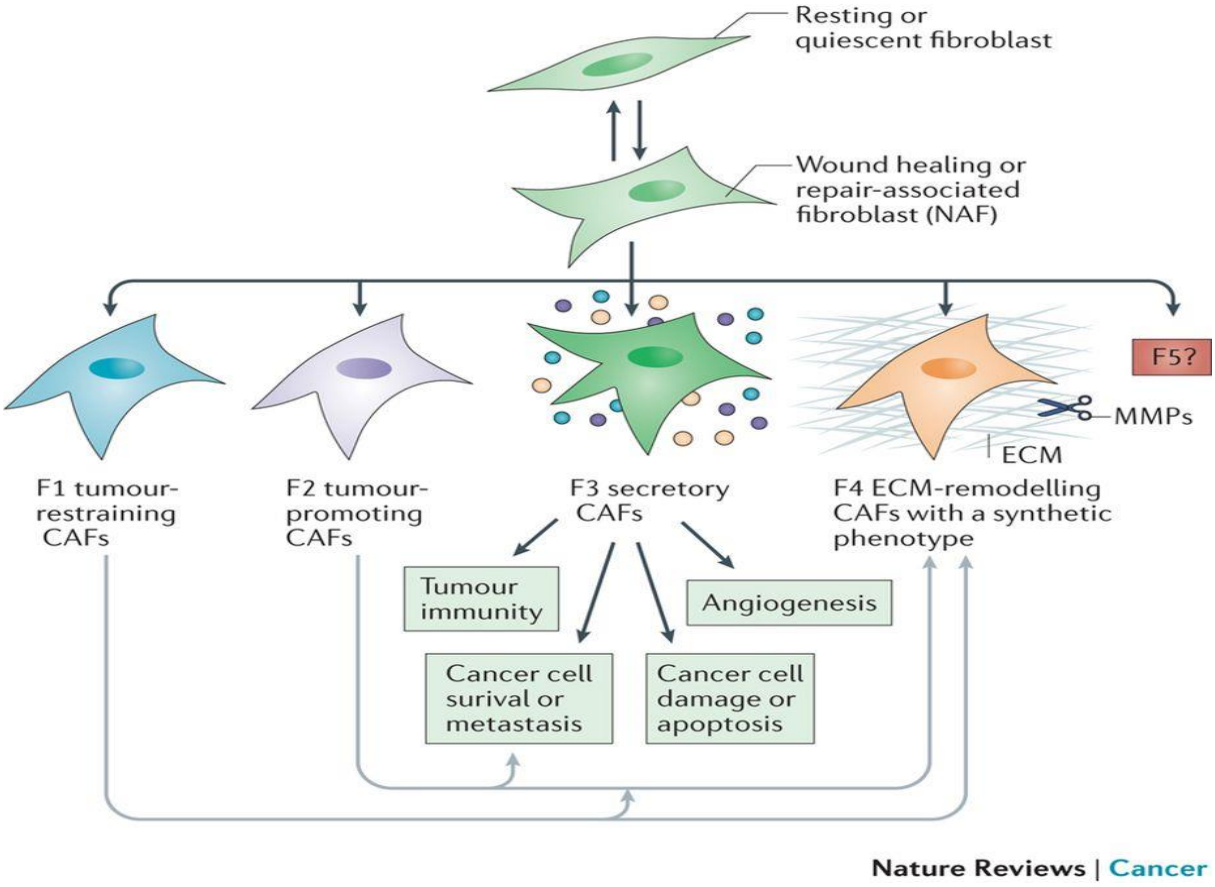

Figure 1.7: CAF subclasses: *It is hypothesized the existents of different subtypes of CAFs within the TME with distinct functions regarding tumor development based on CAFs phenotype. CAFs with the F1 phenotype are thought to have a tumor restraining type, while the F2 type of CAFs have properties enabling this phenotype to promote tumor development. CAFs with F3 phenotype have secretory capabilities and are involved in many aspects of tumor progression. The concept also supports the idea of another type, the F4 CAFs, which are mainly involved in the remodeling of ECM by secretion of different ECM components [86].*

1.4.2 CAFs role in tumor progression

It was originally thought that cancer cells are self-sufficient for tumor progression, but recent studies have revealed stromal cells such as CAFs with essential roles in tumor development [60]. A crucial feature of tumors are the initiation of tumor vasculature in a process known as **angiogenesis**. This provides the rapidly growing tumor with nutrients and oxygen, and also contributes to remove waste products [88]. One of the most pro-angiogenic molecule is vascular endothelial growth factor (VEGF) which regulates growth of new vessels through mechanisms related to Hypoxia inducible factor-1 (HIF-1) [89]. CAFs can support tumor angiogenesis through secretion of VEGF, but also by secretion of PDGF and TGF-β [90, 91]. Stromal derived factor-1 (SDF-1), also known as CXCL12, is also synthesized and secreted by CAFs, which promotes the recruitment of endothelial precursor into tumor stroma, supporting the formation of tumor angiogenesis [92, 93]. IL-6 is a pleiotropic cytokine with a number of pro-tumorigenic activities including angiogenesis through endothelial proliferation and migration [94], and CAFs are an important source for stromal IL-6 [95, 96].

Another key aspect of CAFs in support of tumor progression is through **ECM** remodeling [97]. ECM is a non-cellular but physiologically active component of the tumor stroma, and is important for cell-cell communication, cell adhesion and cell proliferation [98]. Fibroblasts normally produce stromal ECM components such as fibronectin and I, III and V collagens in addition to matrix metalloproteinases (MMPs) for assembly and degradation of ECM [99]. CAFs, due to their activated nature, are engaged in an abnormal production of ECM components and produce increased levels of fibronectin, collagen I and MMPs. Such changes in ECM components can support cancer cell migration and invasion [100]. Metastasis is a hallmark of cancer and is the process were cancer cells are able to detach from the primary tumor site, enter the circulation and develop secondary tumors [101]. CAFs support cancer cell metastasis by initiation of angiogenesis and altering the ECM by mechanisms mentioned above [102] **(Figure 1.8)**.

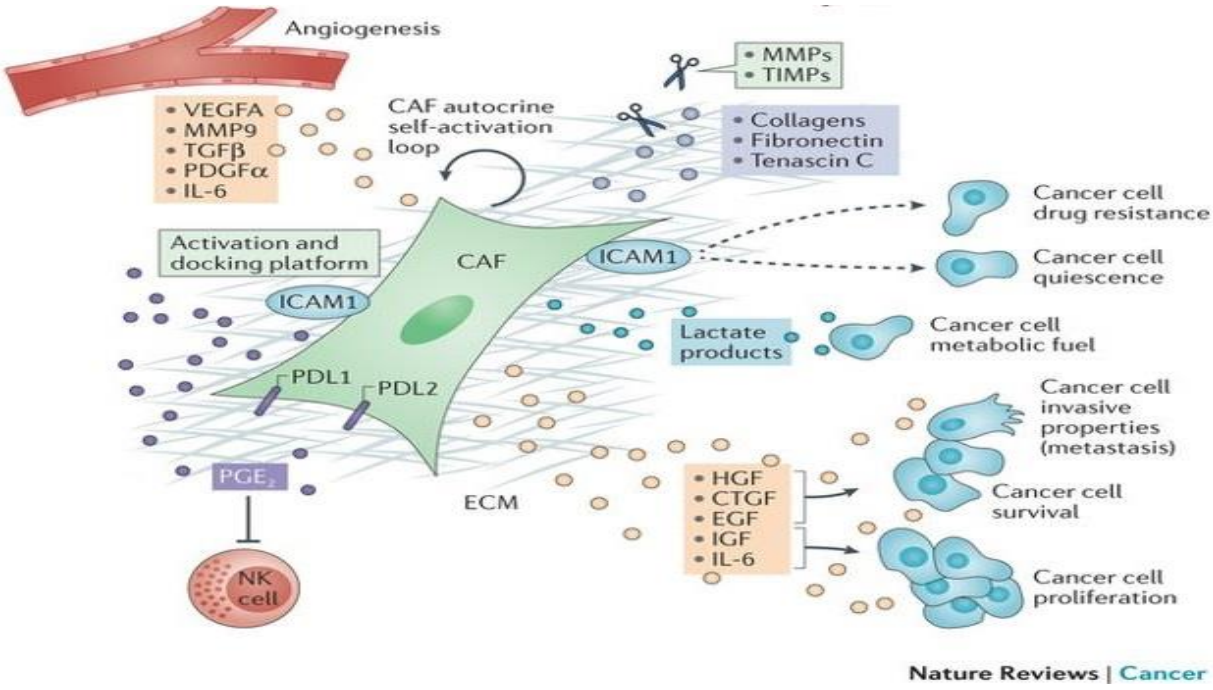

Figure 1.8: CAFs role in tumor progression: *CAFs are an important contributor in the TME by secretion of different factors. Angiogenesis is promoted by CAFs by mechanism involving secretion of VEGF and IL-6, which is aided by CAFs remodeling of ECM through secretion of ECM components such as collagens, fibronectin and MMPs [86].*

An important step for cancer cells to metastasize is to migrate from primary tumor location. This is done through a mechanism named epithelial-mesenchymal transition (EMT). The process of EMT is a developmental process were epithelial cells transdifferentiate into mesenchymal cells which enables the cells to migrate to new regions. EMT is silent in normal cells, but is reactivated in cancer [103]. One of the key changes for cancer cell EMT is the downregulation of inter-cellular adhesion molecules E-cadherin. CAFs can support cancer cells EMT by secretion of hepatocyte growth factor (HGF) and TGF-β, which promote paracrine signaling in cancer cells and induces EMT [104, 105]. Some studies have suggested that CAFs have the ability to detach from tumor stroma, enter the circulation and continue tumor support at metastatic sites [91].

1.4.3 CAFs role in anti-tumor immune responses

There is not only a cross-communication between cancer cells and immune cells, but between CAFs and immune cells as well. Although very frequently ignored by immunologists due to their non-hematopoietic origin, CAFs are one of the most important immunoregulatory cells in the TME [106]. CAFs are important producers of immunoregulatory soluble signals, which

include CXCL1, CXCL2, CXCL5, CXCL6/GCP-2, CXCL8, CXCL9, CXCL10, CXCL12/SDF-1 CCL7, IL-1β, IL-6, IL-10, VEGF, TGF-β, IDO, prostaglandin E2 (PGE_2), tumor necrosis factor (TNF), soluble NKG2D ligands or nitric oxide (NO) [107]. The communication between CAFs and different types of immune cells can be seen in **Figure 1.9**. Macrophages is one of the immune cell types found in TME. CAFs secretion of CXCL12/SDF-1, macrophage colony-stimulating factor (M-CSF) and IL-6 stimulate recruitment of monocytes to TME and the differentiation of monocytes into M2-polerized macrophages to support tumor progression [108].

γδ T cells are one of three immune cell subclasses that express antigen receptor and this cell type has different approaches to promote its anti-tumor surveillance. One of the main action of γδ T cells is their cytotoxic activity, which is provided by production of chemokines and components of cytotoxic granules including perforin, granzyme, tumor necrosis factor and TNF-related apoptosis-inducing ligand [109]. γδ T cells can also act as a bridge between the innate and the adaptive immune system by activation of B cells and aid the humoral immunity [110]. Cells of the TME, including CAFs, are able to regulate the proliferation and function of γδ T cells through secretion of TGF-β, PGE_2, adenosine and soluble NKG2D ligands (MICA/B). These signal molecules can polarize γδ T cells from IFN-γ producing cells to IL-17 producing γδ T cells [111]. CAFs can also suppress the activity of NK cells. The different immunological functions for NK cells includes “cross-talk” with DCs, which provides activating stimuli for both NK cells and DC [112]. NK cells have cytotoxic properties and are able to recognize and kill cancer cells through NKG2D receptor, which binds MHC class I molecules on target cells/cancer cells [22]. CAFs are able to decrease NK cell activation and cytotoxic function by secretion of TGF-β, PGE_2 and/or IDO. These molecules affects NK cells by reducing the transcription of DAP12, resulting in a downregulation of NK activation receptors such as NKp30, NKp40 and NKG2D and the expression of perforin and granzyme. It also alters the expression of IFN-γ for NK cells, which is a stimulating factor for effector CD4 T cells [107].

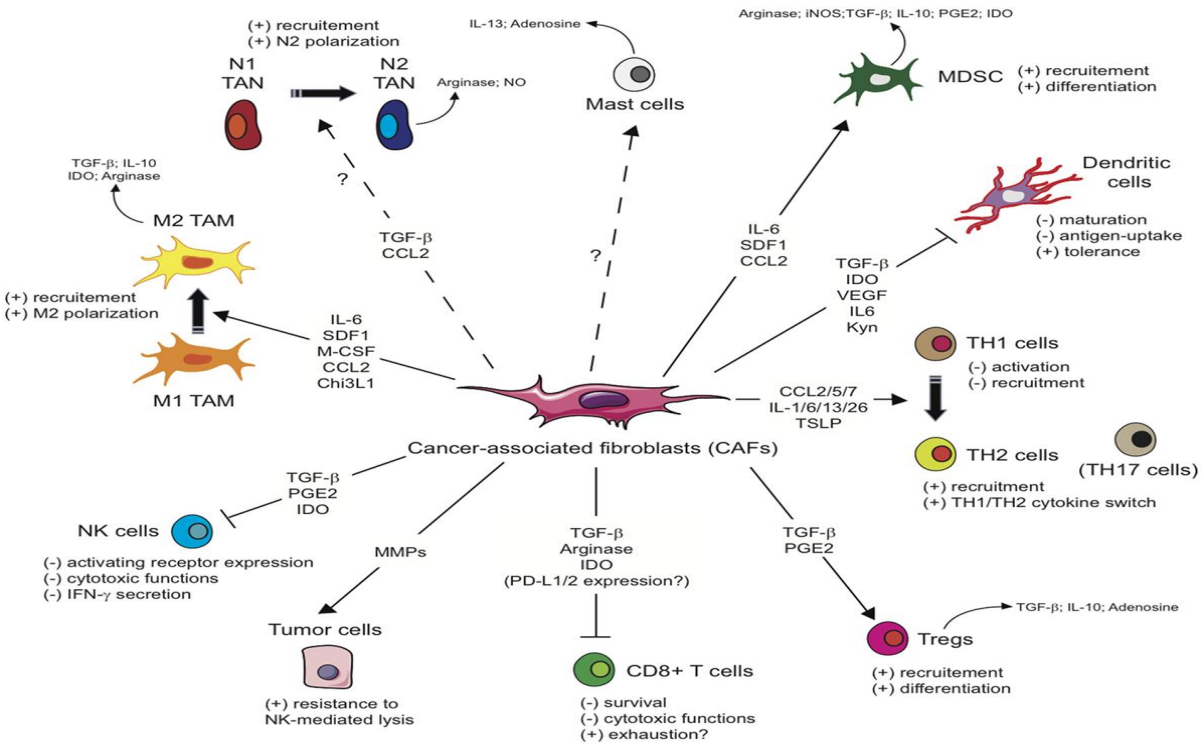

Figure 1.9: Signaling between CAFs and immune cells: *CAFs secrete a broad spectrum of signal molecules, which enables CAFs to "talk" with immune cells within the TME. This communication can alter the function of the immune cells towards pro-tumorigenic phenotypes [107].*

1.5 Dendritic cells

Dendritic cells (DCs) belongs to the innate immune system and are one of the key cellular components regarding immune responses. They can be regarded as a bridge between the innate and adaptive immune system [113]. It was known in the 1960s that lymphocytes exerted the function of the adaptive immune system, but it was not known how lymphocytes become active. Between 1967 and 1973, an exceptional cell type was discovered with the capability to take up antigens, and it was identified that this type of cell, called A cells or the third cell, was responsible for activation of T lymphocytes and antibody related responses. In 1973, Ralph Steinman and Zanvil Cohn unveiled a cell type with phagocytic characteristic and a morphology with dendritic structures. And because of these structures, the cell type was named dendritic cells. It turned out that the same cell discovered between 1967-1973 and the cell type identified by Steinman and Cohn was the same type of cell, the dendritic cell [114].

The majority of cells in the immune system can be found in different functional stages in order to best confront different challenges. This is also the case with DCs, comprising different subclasses to most efficiently encounter, process and transport foreign antigens to lymph nodes

to engage the adaptive immune system. All the different subsets of DCs originate from hematopoietic stem cells. The four major subsets of DCs are; (1) conventional type 1 dendritic cell (cDC1), (2) conventional type 2 dendritic cell (cDC2), (3) plasmacytoid dendritic cells (pDCs), and (4) monocyte-derived dendritic cells (Mo-DCs) [115] **(Figure 1.10)**. From this point on, the focus will be on Mo-DCs since this subset of DCs are used in the study.

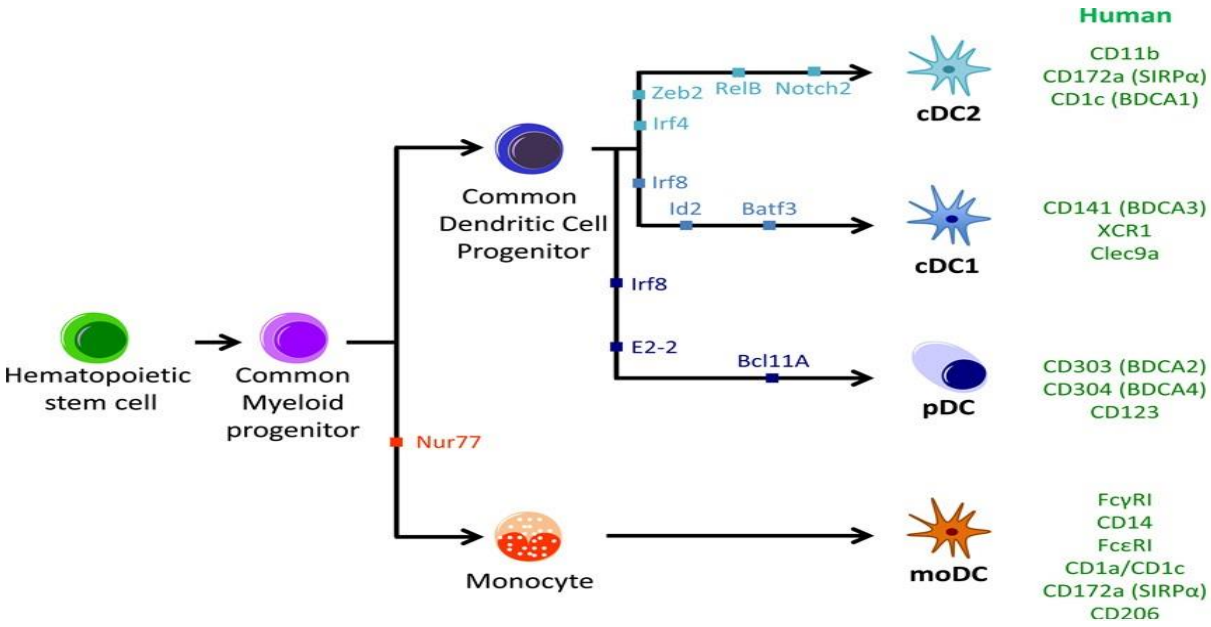

Figure 1.10: Different dendritic cell populations: *DCs belongs to the hematopoietic cell linage, which starts with hematopoietic stem cells (HSC). These cells give rise to the common myeloid progenitors (CMPs), and the transcription factor Nur77 regulates differentiation into monocytes, which can be further development into monocyte-derived dendritic cells (Mo-DCs) during inflammatory conditions. If Nur77 is not activated, CMPs are differentiated by several steps into common dendritic cell progenitor (CDP) which can be further differentiated to conventional type 1 DC (cDC1), conventional type 2 DC (cDC2) and plasmacytoid DC (pDC). Critical transcription factors for development of the different subclasses are showed. Different markers for each subclass can be seen in green [115].*

1.5.1 General biology of DCs

DCs are specialized leucocytes considered as APCs. They are generally found in peripheral tissues and immunological organs, such as the spleen, thymus, bone marrow, lymph nodes and Peyer`s patches [116]. They are part of the primary line of defense for humans with the ability to detect invading bacterial, viral, protozoan and fungal pathogens or other foreign molecules [117]. Macrophages (Mø) are found in all organs and tissues and are also part of our first line of defense. Mø secret IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) upon detection of danger signals, which in turn trigger the transformation of monocytes to immature DC [118]. All nucleated cells in the body are able to present antigens to CD8 T cells

and NK cells through MHC class I molecules deriving from intracellular proteins to initiate immune responses, but DCs have a special ability to take up peptides from the environment, internalize, and present the antigens through MHC class II molecules to CD4 T cells in lymph nodes and launch a full scale adaptive immune response [119].

1.5.1.1 Antigen uptake

iDCs are able to internalize extracellular foreign antigens through different mechanism. This includes receptor-mediated endocytosis, phagocytosis and micropinocytosis [120]. The pathway generating MHC class I and MHC class II molecules with antigens can be seen in **Figure 1.11**. Endogenous proteins are processed by proteasomal proteolysis by proteasomes, which generates peptides able to translocate from cytosol to endoplasmic reticulum (ER). Here, peptides bind to MHC class I molecules, and these peptide-MHC class I complexes are further transported through the Golgi apparatus to the cell membrane for antigen presentation [121]. This pathway is also important for a cross-presentation, which is an important pathway APCs can use to present exogenous peptides through MHC class I molecules to CD8 T cells [122].

iDCs use a broad variety of pattern recognition receptors (PPRs) such as toll-like receptors (TLRs), C-type lectins (CLRs) and nucleotide oligomerization domain-like receptors (NLRs) to detect and capture infectious exogenous non-self-antigens [123]. These exogenous peptides are internalized by endocytosis and processed through a vesicular pathway ending with displaying peptide fragments (antigens) through MHC class II molecules on the surface of mDCs [124]. As with foreign peptides, cancer cells produces pathogen-associated molecular patters (DAMPs) released from dead cancer cells which are recognized as danger signals by iDCs. Death of cancer cells can be caused by hypoxia or nutrient deprivation, which generates host-derived DAMPs, resulting in display of cancer peptides on the surface of DCs [125]. Upon recognition of danger signals by PPRs, iDCs start the maturation process leading to the generation of mDCs. The maturation process can also be initiated by inflammatory cytokines, such as TNF-α and IL-1 [126, 127].

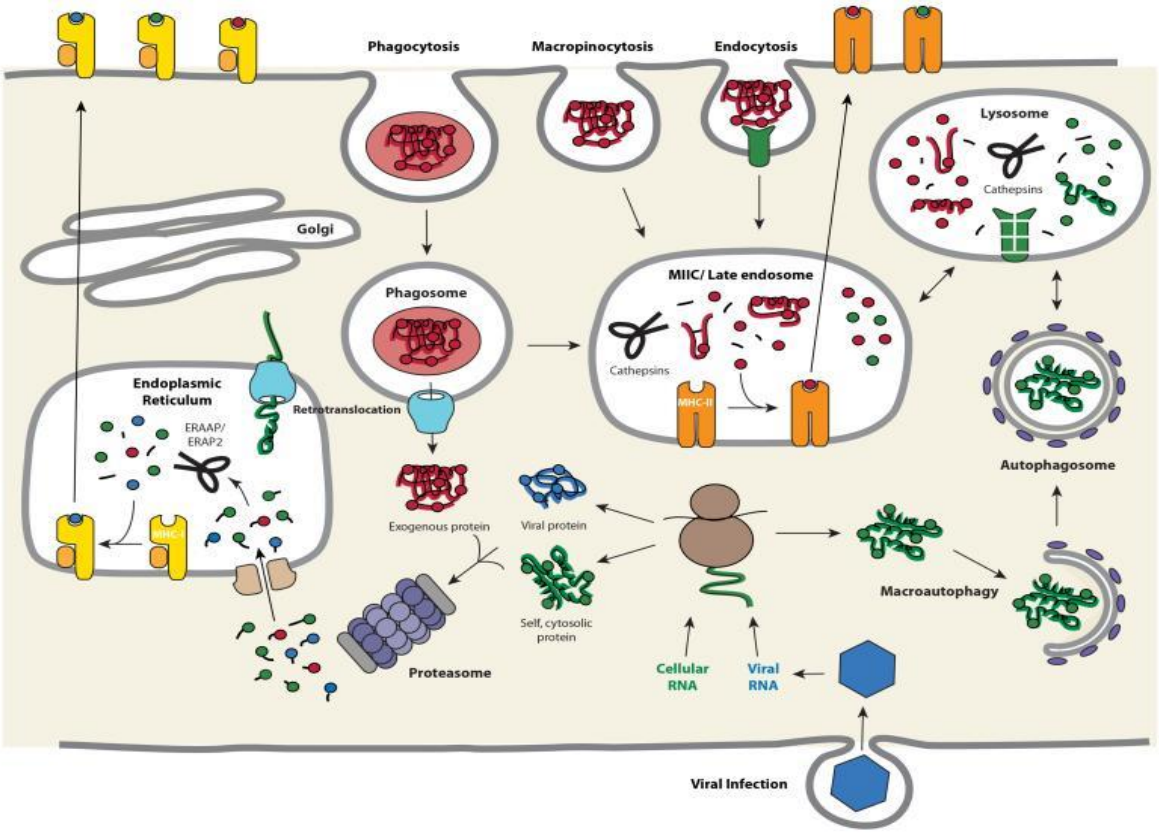

Figure 1.11: Antigen uptake: *Endogenous proteins in cytosol are processed by proteasome generating peptide fragments, which are then translocated to ER. Here, peptide fragments are assembled with MHC class I which are then displayed on the surface of cell with antigen. Exogenous proteins are internalized by iDCs and then processed through late endosomes were peptides are combined with MHC class II molecules and further transported to the cell membrane for display to cells in the adaptive immune system [121].*

1.5.1.2 Migration

While iDCs are specialized in recognition of DAMPs and processing foreign antigens, mDCs are specialized in presentation of internalized peptides to T cells. During the maturation process, DCs modulate the expression pattern of chemokine receptors, and downregulation phagocytosis and endocytosis. The maturation process also involves an upregulated expression of MHC class II molecules, adhesion molecules (CD54) and co-stimulatory molecules CD80 and CD86 for optimizing the properties necessary for T cell priming capacity [128, 129]. One of the key signals for initiation of DC maturation is mediated through TLRs, which results in downstream signaling and activation of nuclear factor-κB (NF-κB), which regulates gene expression of co-stimulatory molecules, MHC molecules and chemokine receptor 7 (CCR7) [130]. Other surface markers are also increased upon maturation of DCs, such as MHC class II (HLA-DR) and CD40 [131]. The cytokine expression is also changed during maturation. iDCs have negative

expression of both IL-10 and IL-12, while mDCs have negative to low expression of IL-10 and medium to high expression of IL-12 [128]. The migration mediated by CCR7 is important for migration of mDCs towards lymph nodes and an illustration of the migration can be seen in **Figure 1.12**. This migration is driven by the chemokines CCL19 and CCL21, which act as ligands for CCR7. These two chemokines are synthesized by lymphatic endothelial cells and T cells in the lymph nodes, helping to guide the mDCs towards its target cells [132].

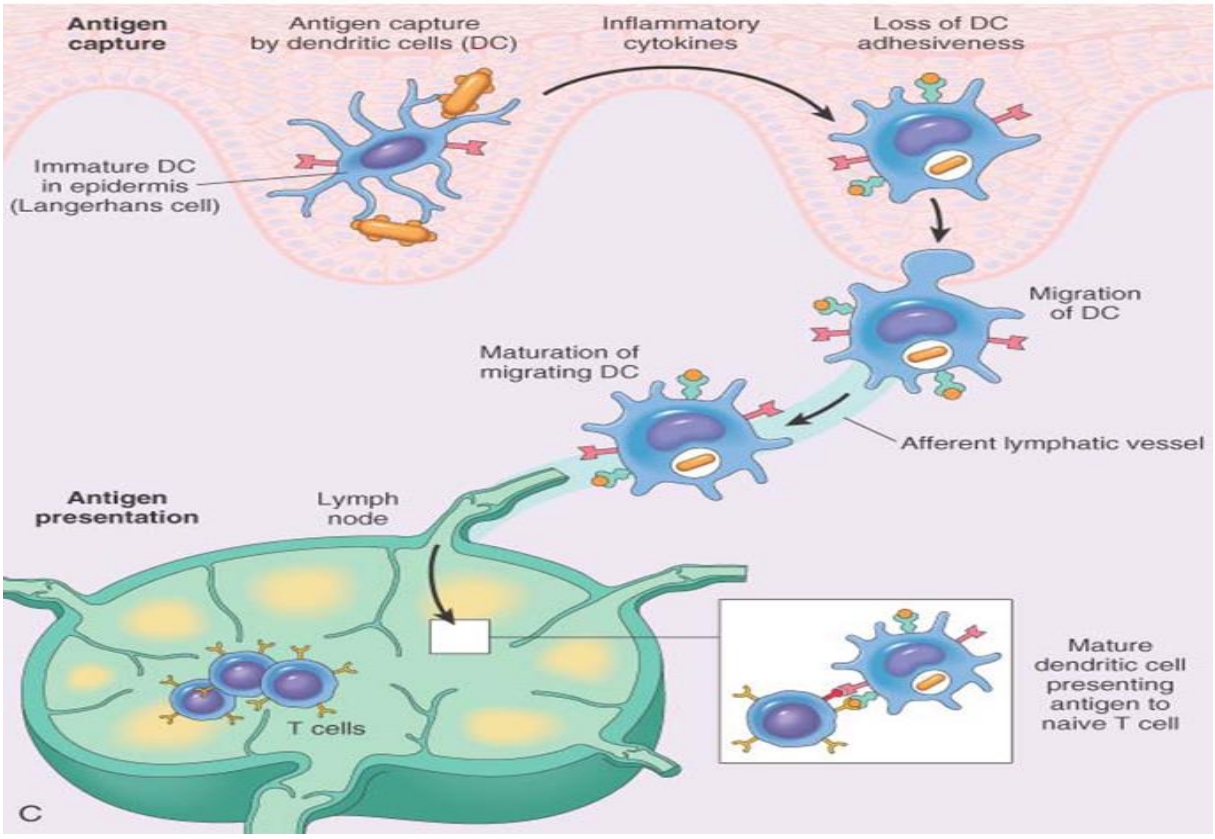

Figure 1.12: Migration of mDCs: *Upon recognition and internalization of foreign antigens, iDCs start to migrate towards lymph nodes driven by an upregulation of chemokine receptors. During the migration, DCs change phenotype towards a mature state, which is specialized in presentation of antigens and activation of naïve T cells in lymph nodes for initiation of an adaptive immune response [133].*

1.5.1.3 Antigen presentation

The **lymph nodes** are comprised of different compartments with their own niche for immune cells. Antigen presenting mDCs migrate towards the paracortex of lymph nodes were they encounter **naïve CD4$^+$ T cells** [134]. Here, mDCs are able to present antigens to naïve CD4$^+$ T cells, which leads to the differentiation of naïve CD4$^+$ T cells to different subclasses of T cells with distinct functions. This will ultimately activate CD8$^+$ T cells with specificity to kill and

eliminate target cells who are displaying antigens similar to the ones presented by mDCs [135, 136]. One mDC is able to interact with as many as approximately 5000 T cells per hour, to illustrate the enormous capacity of mDCs to display antigen to T cells [137]. The full activation of naïve $CD4^+$ T requires two signals; (1) interaction between MHC class II (with antigen) and TCR, and (2) interaction between co-stimulatory molecules CD80/CD86 and CD28, as can be seen illustrated in **Figure 1.13** [117]. mDCs display CD40 on its surface as well, which can be used to interact with CD40L and activate $CD4^+$ T cells. These activated CD4 T cells can further communicate with B-cells and aid the humoral immunity [138].

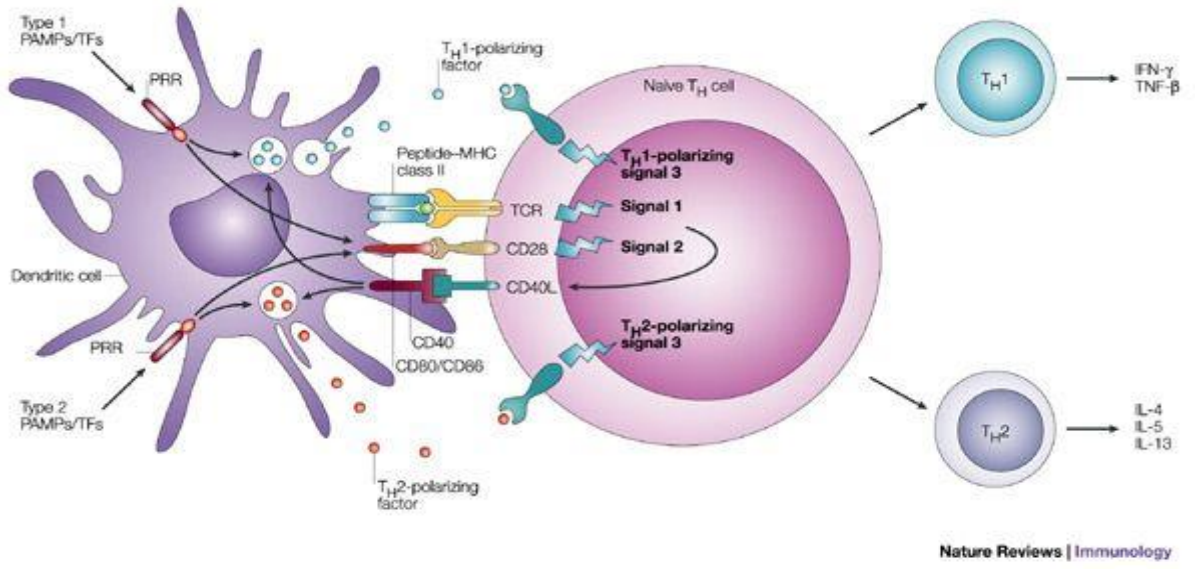

Figure 1.13: Antigen presentation: *mDCs present antigens to naïve $CD4^+$ T cells through MHC class II – TCR integration. This signaling represents signal 1, but the fully activation of naïve $CD4^+$ T cells requires another signal, signal 2, which is provided by interactions between the co-stimulator molecules CD80/CD86 and CD28 [139].*

1.5.2 Dendritic cells in cancer

DCs are the foundation of the "cancer immunity cycle" as illustrated by Chen and Mellman (2013) in **Figure 1.14**. This illustration gives an overview of the function of DCs together with T cells and the cycle involves several steps which eventually lead to the death of cancer cells; (1) cancer cells release antigens due to cancer cell death, (2) which is recognized by DCs and presented to T cells in lymph nodes. (3) This leads to priming and activation of T cells, (4) resulting in migration of T cells to tumor site. (5) Here, tumor-specific T cells infiltrate tumors, (6) and recognize cancer cells by surface antigens (7) leading to the death of cancer cells induced by T cells [17].

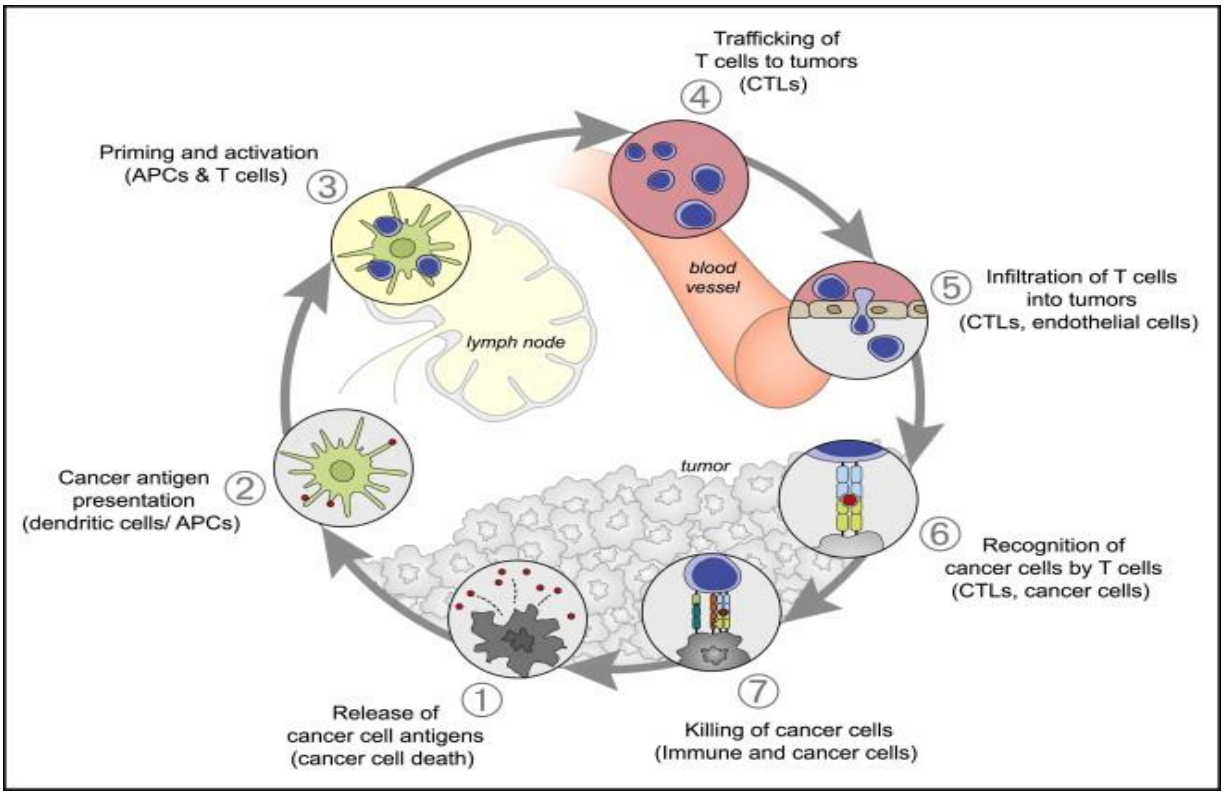

Figure 1.14: Cancer immunity cycle: *DCs and T cells cooperate to kill cancer cells in what is known as the "cancer immunity cycle" [17].*

But, tumors can interfere at all stages in the "cycle" trying to escape the immune system. DCs are mainly involved in the three first steps, and cancer cells can induce either apoptosis of DCs or polarization towards a tolerogenic and immunosuppressive phenotype for immune evasion [140]. Apoptosis can be induced in DCs through mechanisms involving production of tumor-derived gangliosides and HMGB1 [141]. Decoy receptor 3 (DcR3) or tumor necrosis factor receptor (TNFR) have ability to induce apoptosis for DCs as well. The level of DcR3 is elevated in a number of different cancers and is used as a biomarker to predict inflammatory disease progression and cancer metastasis [142]. DcR3 is able to induce apoptosis in DCs through pathways including protein kinase C δ (PKC-δ) and c-Jun N-terminal kinase (JNK), leading to up-regulation of death receptor 5 (DR5) causing recruitment of Fas-associated death domain (FADD) to promote apoptotic signals for DCs [143].

Tumor stroma can also educate DCs to become immunosuppressive, and these DCs are known as regulatory DCs (DCreg). The generation of DCreg from normal DCs is mediated through the broad variety of immunosuppressive factors tumors produce, such as NO, IL-10, IL-6, arginase-I, VEGF, IDO and TGF-β. TI DCs (TIDCs) are found in the TME of many different

types of cancer, such as breast, colorectal, lung, renal, head and neck, bladder, gastric and ovarian [144]. The formation of DCreg prevents T cells from being activated by DCs through antigen presentation followed by interruption of priming and activation of T cell [145]. This affects all T cell mediated immune responses towards tumors.

Tumor cells not only block the activation of T cell-mediated immune response by DCs, but also suppress T cell activity in tumor stroma by the generation of DCreg. Normally, DCreg are important for maintaining the equilibrium needed between inflammatory responses and tolerance. There is a clear distinction between the immature phenotype of DCs with the ability to take up antigens and the mature phenotype for presentation of such antigens. But in regard to DCreg, this is less clear. It was initially demonstrated that iDCs are able to induce tolerance, which could be explained by the fact that iDCs process antigens, and in the absence of co-stimulatory molecules induce T cell anergy and deletion. But newer research has revealed that mDCs can induce immunosuppressive functions as well, which suggest that DCreg is a functional state rather than a special subclass that can be defined by certain phenotypic markers [131].

Tumor-induced DCreg can be characterized by the combination of surface marker expression and cytokine production. This involves markers such as PD-L1, PD-L2, B7-H3, B7-H4, CD103, ILT3/4. Cytokines synthesized and other immunoregulatory factors produced by DCreg include IL-10, IL-1β, TGF-β, IDO, arginase I and iNOS. In addition, DCreg express low levels of CD11c, MHC class II and co-stimulatory molecules CD80 and CD86. By these mechanisms, DCreg are able to affect and alter the function of T cells in all aspects of the "tumor immunity cycle" [146]. By the lack of MHC class II molecules, DCreg are not fitted to provide T cells with the necessary signal 1 for activation or signal 2 provided by co-stimulatory molecules CD80/CD86 for full T cell activation. Other surface receptors, such as the inhibitory ligands B7-H3, B7-H4, can bind to unknown receptors on T cells and reduce the proliferation of T cells [147]. **Figure 1.15** gives an overview of receptors found on APCs and T cells regarding T cell mediated immune responses.

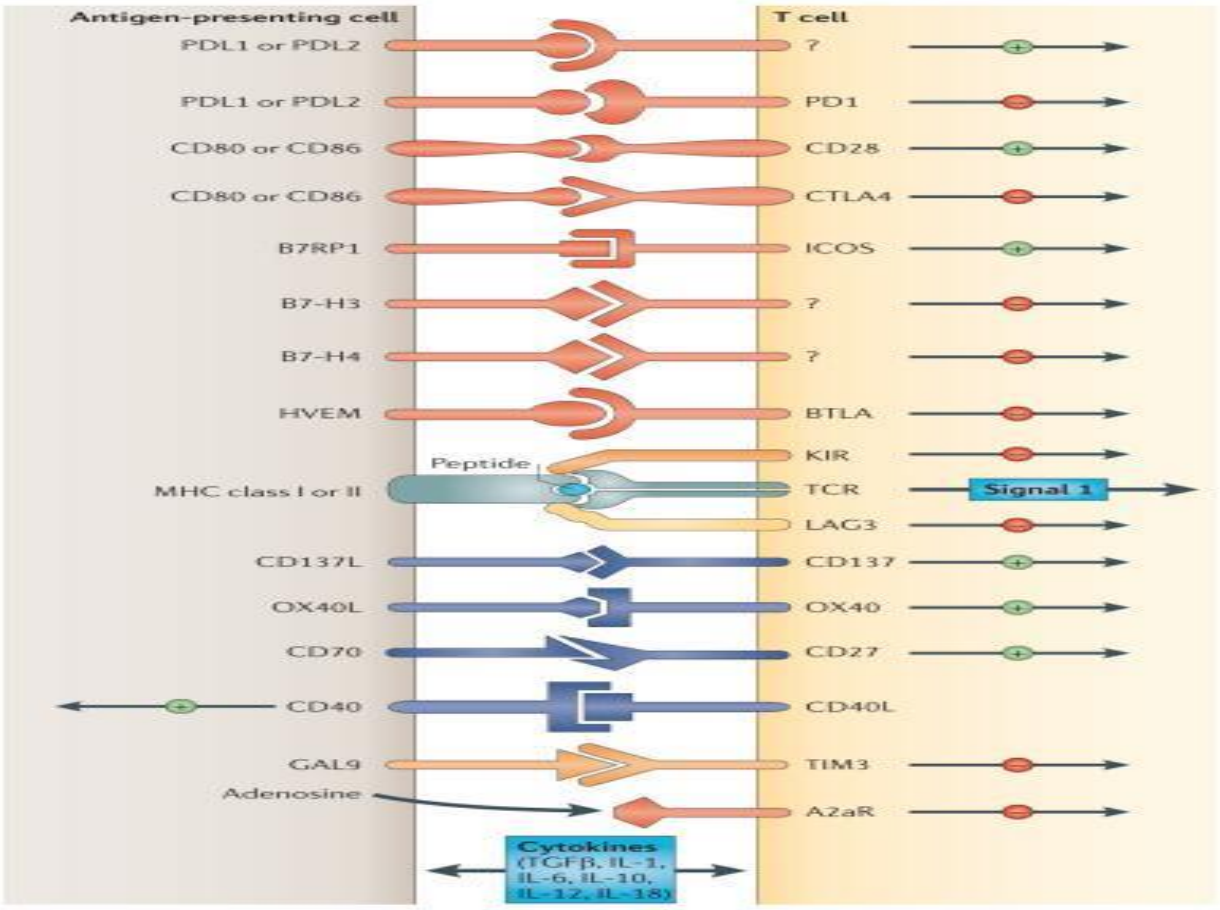

Figure 1.15: Co-stimulatory and inhibitory receptors mediate T cell responses: *Antigen-presenting cells express both co-stimulatory and inhibitory receptors on their surface to mediate different T cell responses [148].*

1.6 Radiation and the tumor microenvironment

There are many different types of cancer treatments. This includes cytostatic chemotherapy, radiotherapy (RT), immunotherapy, hormone therapy, targeted therapy and surgery or a combination of these treatments [149-153]. Over 50 % of all diagnosed cancer patients go through RT during the course of disease, which makes RT one of the most used treatment modalities in oncology [154]. Treatment regarding RT was first used to target and kill cancer cells themselves because of the thought that only cancer cells contributed to the development of disease. In recent years, the understanding that tumors are not solely dependent on cancer cells, but also surrounding stromal cells, has led to new strategies involving RT where TME is the target for RT as well [155]. RT is able to trigger anti-tumor effects beyond the direct killing of malignant cells. On a molecular level, RT is able to induce non-reparable damage to cell DNA and this results in cellular stress and apoptosis. The use of RT can also affect leukocytes, since the radiation field often includes thymus, hematopoietic bone marrow or large volume of

blood [156]. Not only does RT affect the intended area, but other localizations as well. This phenomenon is known as the abscopal effect and can be thought of as an systemic anti-tumor immune response [157].

1.6.1 Effect of radiotherapy on antitumor immunity

When RT is applied locally, affected cells die and releases immunogenic factors through a process known as immunogenic cell death (ICD) [158]. ICD is characterized by the increase of endogenous DAMPs, which includes calreticulin (CRT), HMGB-1 and ATP [159]. DCs are activated by elevated levels of DAMPs, which in turn activates T lymphocytes through antigen presentation. Both HMGB-1 and ATP are able to act directly on DCs through binding of Toll-like receptor 4 (TLR-4) and purine receptor, respectively [160]. Binding of HMGB-1 stimulates production of cytokines TNF, IL-1, IL-6 and IL-8 by monocytes and also enhances DCs antigen presentation by preventing degradation of antigens within DCs, while binding of ATP leads to synthesis of IL-1β. The release of chemokines CXCL10 and CXCL16 as a response to RT can induce migration of T cells to tumor stroma. It has also been reported that low-dose RT can mobilize NO expressing macrophages to tumors with the ability to stabilize tumor vasculature [161] **(Figure 1.16)**.

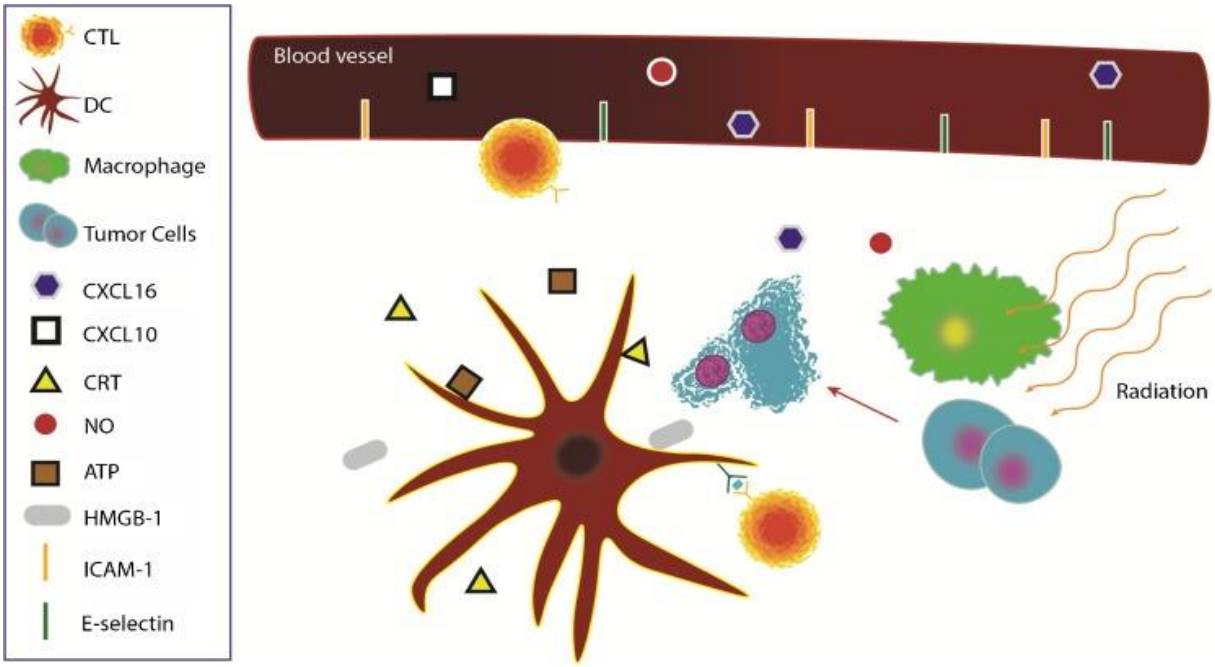

Figure 1.16: Effect of radiotherapy: *Immunogenic cell death is induced by RT, which results in release of DAMPs, such as HMGB-1, ATP and calreticulin. DAMPs generates an immune response by binding to DCs, leading to activation of cytotoxic T lymphocytes (CTL). RT also triggers production of chemoattractants CXCL10 and CXCL16 causing migration of T cells to TME [161].*

1.6.2 Effect of RT on TME

The effects of RT on tumor vasculature depends on some factors. How tumor blood vessels react to RT depends on number of fractions, dose rate, total radiation dose and fraction size [162]. Additionally, quiescent endothelial cells are more resistant to RT compare to proliferating endothelial cells and there is evidence supporting rapid death of vasculature initiated by singe high-dose irradiation, and also reduced vascular density with hyper fractionated irradiation [163]. The induced apoptosis for vascular endothelial cells as a result from RT contributes to the starvation of cancer cells and the elevation of hypoxia. The elimination of vasculature for tumors can result in a hypoxic and acidic tumor microenvironment with limited nutrition. This can cause indirect death of cancer cells [162].

CAFs, being one of the most abundant cell types in tumor stroma, are also affected by RT. Normal fibroblasts and CAFs are relatively radioresistant cells and are able to withstand RT doses above 50 Gy. But there are *in vitro* studies demonstrating that RT doses >10 Gy induces an irreversible state of senescence for fibroblasts, and low-dose RT results in reversible DNA damage without growth arrest. Fibroblasts in a senescence state synthetize proteolytic enzymes, cytokines, growth factors and reactive oxygen species (ROS) able to cause a pro-tumorigenic environment [164]. Another study demonstrated that immunosuppressive molecules secreted by CAFs, such as PGE_2, IL-6, IL-10 and TGF-β remain unchanged after RT of CAFs [165]. Knowledge about the effect irradiation has on CAFs immunosuppression is still poor. In this study, we wanted to investigate the effect of RT on CAF-mediated immunomodulation regarding DCs.

2 Aim of study

Cancer-associated fibroblasts are one of the most abundant components found in the tumor microenvironment. Studies have demonstrated that radiotherapy could lead to permanent phenotypic changes for the CAFs. Immune cells recruited to the TME can be "educated" by CAFs to gain immunosuppressive phenotypes creating a pro-tumorigenic TME, but the mechanisms behind this alteration is still poorly understood

The purpose of this study was to investigate the influence of RT on CAFs modulation of DCs phenotype and functions in an *in vitro* setting. The main objective was divided in the following sub-aims:

- Characterize potential effects of CAFs on DCs phenotype differentiation/maturation
- Characterize potential effects of CAFs on DCs immune functions
- Determine whether irradiation alters CAF-mediated immunoregulation of DCs

3 Material

3.1 Cell culture medium and supplements

	Catalog number	Supplier	Origin
Classical Medium	-	-	-
Dulbecco`s Modified Eagle Medium (DMEM)	D5796	Sigma-Aldrich	USA
Roswell Park Memorial Institute (RPMI)	R8758	Sigma-Aldrich	USA
Bronchial Epithelial Basal Medium (BEBM)	CC-3171	Lonza	Belgium
Supplements	-	-	-
Fetal Bovine Serum (FBS)	S0115	Biochrom	Germany
Insulin-Transferrin-Selenium	25-800-CR	Corning	USA
Sodium Pyruvate (100 mM)	11360070	Gibco	UK
Penicillin-Streptomycin	P4333	Sigma-Aldrich	USA
L-ascorbic Acid	A92902	Sigma-Aldrich	USA
Accutase Cell Detachment Solution	561527	BD Biosciences	USA
Complete DMEM growth medium			
DMEM with 10 % FBS + Penicillin-Streptomycin + L-ascorbic acid			
Complete RPMI growth medium			
RPMI + 10 % FBS + Penicillin-Streptomycin + Pyruvate			
CAFs Freezing medium			
RPMI + 60 % FBS + 10 % DMSO			
PBMC/Lymphocytes/dendritic cells Freezing medium			
FBS + 10 % DMSO			

3.2 Cell Culture Reagents

	Catalog number	Supplier	Origin
Cell Detachment	-	-	-
Enzyme Free Cell Dissociation Solution PBS Based	S-014-B	Millipore	Norway
Trypsin-EDTA solution	T4049	Sigma Aldrich	USA
Cell Freezing	-	-	-
Dimethyl Sulfoxide (DMSO)	20385.01	Serva	Germany
PBMC Isolation	-	-	-
Lymphoprep™	1114544	Axis-Shield	Norway
Cell Washing	-	-	-
Phosphate Buffer Saline (PBS)	D8537	Sigma Aldrich	Germany

3.3 Supplies

	Catalog number	Supplier	Origin
Plastic ware	-	-	-
NuncEasyFlask 25cm^2	163371	ThermoScientific	Denmark
NuncEasyFlask 75cm^2	156499	ThermoScientific	Denmark
NuncEasyFlask 175cm^2	159910	ThermoScientific	Denmark
Falcon Tissue Culture Dish Sterile	353003	Corning	USA
Falcon 6-well plates TC-treated	353046	Corning	USA
Falcon 24-well plates TC-treated	353047	Corning	USA

3.4 Antibodies and recombinant proteins

	Catalog number	Supplier	Origin
Human IL-4	200-04-50UG	PeproTech	USA
Human GM-CSF	300-03-50UG	PeproTech	USA
Human IL-1β	200-01B-50UG	PeproTech	USA
Human TNF-α	300-01A-50UG	PeproTech	USA
Human IL-6	200-06-50UG	PeproTech	USA
Prostaglandin E_2	P5640	Sigma-Aldrich	Norway
Human CCL19 (MIP-3β)	130-105-744	Miltenyi Biotec	USA
Human anti-CD1a	130-111-875	Miltenyi Biotec	USA
Human anti-CD14	130-110-522	Miltenyi Biotec	USA
Human anti-CD209	130-099-727	Miltenyi Biotec	USA
Human anti-CD80	130-101-213	Miltenyi Biotec	USA
Human anti-CD86	130-116-159	Miltenyi Biotec	USA
Human anti-CD40	130-110-946	Miltenyi Biotec	USA
Human anti-HLA-DR	130-111-797	Miltenyi Biotec	USA

3.5 MACS cell separation products

	Catalog number	Supplier	Origin
MACS Multistand	130-042-303	Miltenyi Biotec	USA
MidiMACS separator	130-042-302	Miltenyi Biotec	USA
CD14 Microbeads	130-091-097	Miltenyi Biotec	USA
CD4 T Cell Isolation Kit	130-094-131	Miltenyi Biotec	USA
LS Columns	130-042-401	Miltenyi Biotec	USA
MACS BSA Stock Solution	130-091-379	Miltenyi Biotec	USA
autoMACS™ Rinsing Solution	130-091-222	Miltenyi Biotec	USA

3.6 FACS flow product

	Catalog number	Supplier	Origin
Reagents			
FACS Flow	342003	BD Biosciences	Canada
FACS Rinse	340346	BD Biosciences	Canada
Tubes			
Falcon Polystyrene Round-Bottom tube 5 ml	352054	Corning	USA

3.7 Other supplies

	Catalog number	Supplier	Origin
25cm Cell Scraper	734-2602	VWR	UK
Stainless sterile blade	90010-23	Paragon	USA
Falcon tube 15 ml	734-0451	VWR	UK
Falcon tube 50 ml	734-0448	VWR	UK
BD PlastiPak Syringe	302188	ThermoScientific	USA
Syringe Filter Unit	Z355518-50EA	Millex	Norway
Pasteur pipette 7ml sterile	LSUK726128	VWR	UK
Nunc CryoTube Vails	363401	ThermoScientific	Denmark
Neubauer Hemocytometry	68052-14	Electron Microscopy Sciences	USA
FITC dextran	60842-46-8	Sigma-Aldrich	Norway
CellTrace™	C34557	ThermoScientific	Denmark

3.8 Instruments

	Supplier	Origin
Heraeus Miltifuge X3 Centrifuge	ThermoScientific	Germany
Eppendorf 5417R centrifuge	Eppendorf	Germany
Grant Sub Aqua Pro Water Bath	VWR	UK
Heraeus Function Line UT12P Heating Oven	ThermoScientific	Germany

Heracell 150	ThermoScientific	Germany
Heracell 150i	ThermoScientific	Germany
Nikon Eclipse TS100	Nikon	Thailand
Incubating Mini Shaker	VWR	UK

3.9 Kits

	Catalog number	Supplier	Origin
Human IL-12 DuoSet®	DY1240-05	R&D Systems	USA
Human IL-10 DuoSet®	DY217B-05	R&D Systems	USA

3.10 Elisa reagents

	Catalog number	Supplier	Origin
Reagent Diluent concentration 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)	100731	Millipore	Germany
HEPES	H3784	Sigma-Aldrich	USA
Sodium Hydroxide (NaOH)	71690	Sigma-Aldrich	USA
Wash Buffer	WA126	R&D Systems	USA
Reagent concentration 2	DY995	Sigma-Aldrich	USA
Normal Goat Serum	DY005	R&D Systems	USA
Tris	72H5601	Sigma-Aldrich	USA
Sodium Chloride	K26478104917	MERCK	Germany

3.11 Software	Supplier	Origin
CellQuest™ Pro Software	BD Bioscience	USA
Flow Jo Office V10	Tree Star	USA
BD FACSdiva™ Software	BD Bioscience	USA
Ascent Software	Thermo Electron Corporation	USA
Bio-Plex Manager™ 6.0 Software	BIO-RAD	USA

SoftMax Pro Software	Molecular Devices	USA
Excel 2018	Microsoft	USA
GrapPad Prism 7	GraphPad	USA
Spot Software 4.7	Diagnostic Instruments Inc.	USA

4 Methodology

The following sections describes the methods used in the study. Flowchart presented in **Figure 4.1** gives an overview of methods and direction in the course of the study.

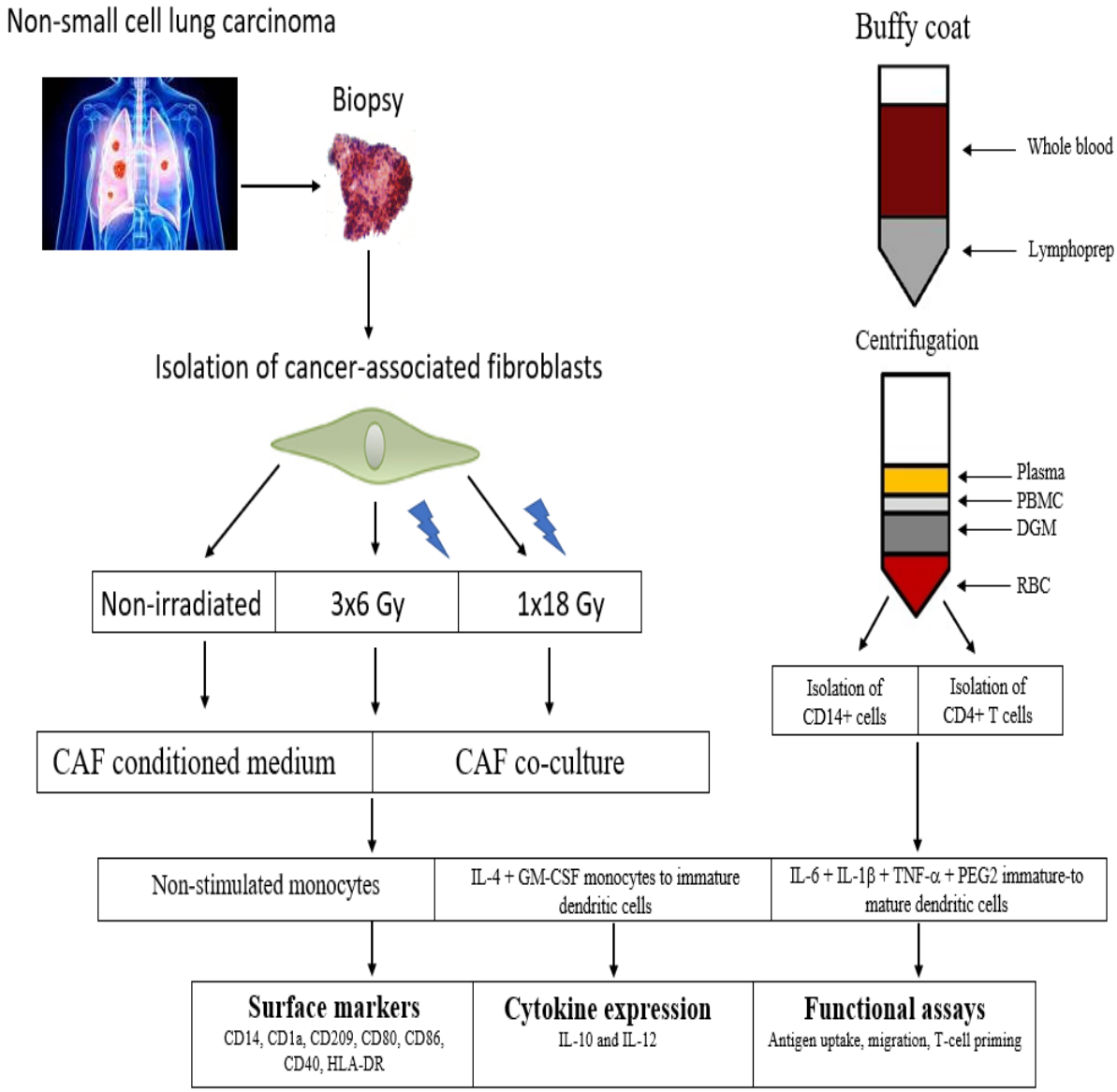

Figure 4.1: Flowchart of the study: *CAFs were isolated from tumor specimens resected from patients with NSCLC and expanded in monolayers. Buffy coats were used for isolation of PBMCs, which was further used to isolate naïve $CD4^+$ T cells and $CD14^+$ monocytes. Monocytes were differentiated into iDCs and mDCs followed by incubation with non-irradiated or irradiated CAF-CM/co-culture with CAFs. The immunomodulatory effects of CAFs on DCs was explored by analysis of surface marker assays, cytokine expression with IL-10 and IL-12 and functional assays (antigen uptake, migration and priming)* ***(PBMC=peripheral blood mononuclear cells, DGM=density gradient medium, RBC= red blood cells)***

4.1 Ethical statement

All blood samples from healthy individuals and tumor specimens from patients with NSCLC were anonymously obtained and written consent was given from all individuals in accordance with the Declaration of Helsinki. This study is approved by the Regional Ethical Committee (REK# 2016/2307, 2016/714, 2014/40). Methods involving the use of human material was done following the proper regulations and guidelines.

4.2 Biological samples and Patients

Human tumor samples were obtained from four different patients with NSCLC at Tromsø University Hospital of Northern Norway, and information regarding donors is described in **Table 4.1**. Patients used in the study had not received any additional therapy before tumor biopsy samples were collected. Concentrated leucocyte blood samples (buffy coat) were received from unrelated healthy donors from the University Hospital of Northern Norway blood bank.

Table 4.1: Overview of tumor sample donors

Number	Age	Sex	Tumor Type	T-size (mm)	T-stage	N-stage
Donor 1	71	M	Squamous cell carcinoma	35	2a	0
Donor 2	68	M	Squamous cell carcinoma	22	1c	0
Donor 3	71	F	Adenocarcinoma	25	1	0
Donor 4	65	M	Squamous cell carcinoma	30	3	0

Abbreviations: M=Male, F=Female
N-stage=0: Tumor has not spread to nearby lymph nodes

4.3 Isolation and culture of cancer-associated fibroblasts from NSCLC

4.3.1 Selection of cell source: Primary cells

Our laboratory chose to work with human primary cells cultures (cells directly isolated from human tissue and cultured *in vitro*) over cells lines (immortalized cells) because the primary cells display closer phenotypic characteristics similar to the original tissue. This enables more accurate comparison between *in vitro* data to *in vivo* circumstances in humans [166]. Cultured

tumor tissue fibroblasts have a restricted proliferation capacity and can ultimately reach the state of replicative senescence (permanent growth arrest). Fibroblasts from already established cells lines have transformed phenotypes with frequently chromosomal aberrations resulting in abnormal cells with unlimited proliferation capacity [167]. When working with human primary cell cultures, a limitation one may experience is change of original features of the primary culture each time cells are passaged (subcultures). Because of these changes, it is important to use cells that have been expanded for short periods and the same number of times to ensure that the result from experiments are similar.

4.3.2 Isolation and cell culture procedures

Human biopsy samples were collected from the pathology department of Tromsø University Hospital within few after surgical resection from patients with NSCLC. Tubes with tumor biopsies were sterile and contained Dulbecco`s Modified Eagle`s Medium (DMEM). Samples were processed quickly to make sure biopsy specimen were in best condition as possible for further studies.

The four following steps were done to ensure the correct isolation and culturing of primary NSCLC CAFs:

- Tissue digestion
- Enzyme-free detachment of cells
- Cell passage of CAFs
- Cryopreservation of primary tumor CAFs

Tissue digestion

Tissue was received in a 50 mL falcon tube. Solution from falcon tube was extracted, leaving just enough solution to cover the biopsy sample. Solution with tissue were then poured into a sterile petri dish and cut in smaller pieces (1-1.5 mm^3) with surgical blades. Tumor biopsy pieces were put together within 5 mL of 400 IU - Accutase (Dulbecco's PBS (0.2 g/L KCl, 0.2 g/L KH2PO4, 8 g/L NaCl, and 1.15 g/L Na2HPO4) containing 0.5 mM EDTA (4Na and 3 mg/L Phenol Red) and transferred to a T-25 cell culture flask and subjected to enzymatic digestion for 60 minutes by placing the T-25 flask on an incubating mini shaker at 37ºC. Afterwards, tumor tissue samples were eagerly shaken to redeem cells in the pieces. Tumor fragments were then collected in a sterile 15 mL falcon tube and centrifuge for 3 minutes at 400 x G to eliminate

collagenase. Supernatants were discarded, and pellets resuspended in 12 mL of fresh complete CAF growth medium containing DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin. Then, 2 mL of cell suspension was added in each well in 6-well tissue culture plate. Tumor pieces were divided equal amongst the wells and the plate was placed in a cell culture incubator at 37ºC with low oxygen (3% O_2), which allowed cells to become attached to the solid substrate.

CAFs isolation

Each well contained a mixture tumor-derived cells the day after tissue digestion, primarily tumor cells and fibroblasts. For fibroblast enrichment, medium from all 6 wells was first removed and 2 mL of pre-warmed Phosphate Buffered Saline (PBS) were then filled and kept in each well for 1-2 minutes. Afterwards, PBS were removed and replaced with Enzyme-Free solution, which promotes selective detachment of fibroblasts while maintaining the structural integrity of cell surface proteins. Plates were placed in culture incubator at 37ºC low oxygen (3% O_2) for 10 minutes. Wells were afterwards examined with microscope to validate CAFs detachment from wells. If observations with microscope confirmed that CAFs remained attached, another 500 μL of pre-warmed Trypsin-EDTA (0.25 % - 1mM) solution was added to each well for 1 minute to facilitate further detachment of CAFs by cutting away focal adhesion molecules enabling fibroblasts to anchor to the plate surface. Detached CAFs were collected in a 15 mL falcon tube and centrifuged for 7 minutes at 350 x G and then resuspended in 10 mL of growth medium (DMEM + 10 % FBS). The 10 mL cell suspension was divided between two T-75 culture flasks and additional 5 mL of growth medium were added to each T-75 flask with a total volume of 10 mL in each T-75 flask. The flask was then placed in cell culture incubator cabin at 37ºC in a 5 % CO_2 humidified atmosphere for further proliferation of CAFs. Importantly, CAFs were incubated in serum-enriched medium which selectively promotes the expansion of fibroblasts while kills tumor cells.

Passaging of fibroblasts

The CAF cultures were observed with microscope every 24 hours, and the growth medium was changed every 2-3 days until the CAFs reached an optimal confluence between 80-90 %. This is important, since CAFs could die without space to grow. The morphology of fibroblasts can be described as an elongated shape, which are anchored-dependent. The result of this is that CAFs grow in a monolayer while they are attached to a solide or semi-solide substrate, as can be seen in **Figure 4.2**.

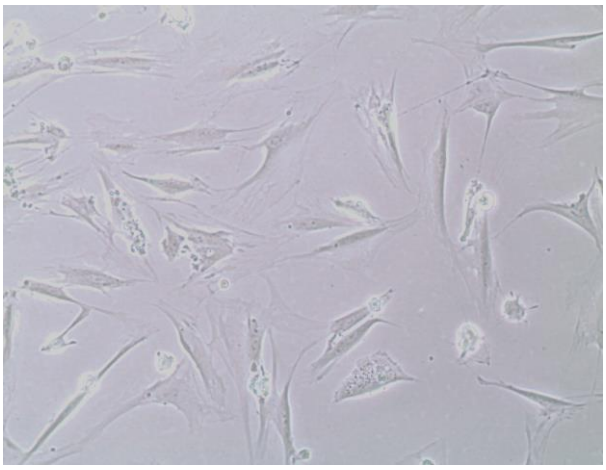

Figure 4.2: Fibroblasts cultured in T-75 flask: *Isolated fibroblasts from a patient with NSCLC with the characteristic elongated morphology attached to the surface in a monolayer. Pictures were taken with an inverted microscope connected to SPOT 4.7 image software.*

The medium in T-75 flask was discarded when the fibroblasts reached the desired confluency of 80-90 % and replaced with Enzyme-Free detachment solution. This solution was kept in the flask for 10 minutes at 3 % O_2 followed by adding Trypsin-EDTA (0.25 % - 1mM), which was kept in the flask for 2 minutes at 3 % O_2 to desegregate CAFs clusters. Cells were collected in a 15 mL falcon tube and centrifuged at 350 x G for 10 minutes, supernatant discarded and pellet resuspended in 10 mL of growth medium and divided between two T-175 flasks with a total volume of 20 mL in each flask. Flasks were incubated at 37ºC in a 5 % CO_2 humified atmosphere. To avoid extensive cell de-differentiation and potential induction of cell senescent by replication, all experiments were performed with cultures at passage 3-4 and no longer.

Cryopreservation of primary tumor fibroblasts

After 2nd passage of CAFs, the cells were used for further analyses or cryopreserved for use in later experiments. Cells destined for cryopreservation were first detached with Enzyme-Free solution and Trypsin-EDTA (0.25 % - 1mM) solution as previously described. Fibroblasts were then counted with a Neubauer Hemocytometry, centrifuged and cryopreserved at 80ºC in freezing medium consisting of DMEM with 10 % Dimethyl sulfoxide (DMSO) and 90 % FBS with 2-3x10^6 cells per mL per CryoTube. DMSO is one of the most preferred types of cryo-protectants used in laboratories to prevent crystals to be formed during the freezing process of cells, which can damage the cell membrane. But, it is important to remember that DMSO can be toxic for cells when used at higher concentration (< 10 %) or if cells are exposed to DMSO for a longer time. Because of this, it is important to eliminate DMSO during the thawing process of cryo-preserved cells as soon as possible. When a CryoTube with cells was taken out from the freezer, the CryoTube was thawed between the hands fast to rapidly thaw the cells. The solution with cells in the CryoTube were then added to a tube with pre-warmed growth medium.

Fibroblasts were then centrifuged at 350 x G for 10 minutes, supernatant (containing freezing medium) discarded and pellet with fibroblasts resuspended in fresh growth medium followed by transferring cells to T-25 flask and place in cabin with 37ºC in a 5 % CO_2 humidified atmosphere and incubated for 24 hours for attachment to the flask. The medium in flask was changed the next day with fresh DMEM medium to ensure that DMSO was completely removed.

4.4 Irradiation and preparation of cancer-associated fibroblasts conditioned medium and CAF co-culture assays

4.4.1 General principles

The condition medium (CM) from non-irradiated and irradiated CAF cultures were collected to investigate the paracrine effects on DCs. This medium contained different mediator substrates such as growth factors and cytokines secreted to the medium by CAFs which could affect different aspects of the immunological functions of DCs.

4.4.2 General procedure

When CAFs were passaged for the second time, cells were seeded at $4x10^5$ cells per T-75 flask and then incubated for 24 hours for attachment to the surface at 37ºC in a 5 % CO_2 humified atmosphere. After the initial attachment and when CAFs reached a confluence about 60-70 %, which took about 2 – 3 days to reach (dependent on donor), CAFs were irradiated. This was done with a Varian clinical linear-accelerator (Department of Irradiation Therapy in Tromsø University Hospital) (**Figure 4.3)**, which produced the high-energy photons of 15 megavoltage (MV). Irradiation doses were delivered to CAFs either as three fraction dose of 6 Gy (24 hours between each fraction) or as a single dose of 18 Gy.

Figure 4.3: Varian clinical linear-accelerator: *T-flask with CAFs were placed on top of tissue-equivalent plates (30 mm depth) with gantry position beneath the T-flask.*

T-flask were placed in the middle of the 20x20 cm field size and on top of three 10 mm thick water/tissue-equivalent Perspex-plates (30 mm in total), since photons produced at 15 MV reach the maximum dosage at 30 mm depth. The Gantry was positioned beneath the field and transported the beam from the linear-accelerator to the target.

After irradiation, the T-flask with irradiated CAFs was placed back in incubator with 37ºC in a 5 % CO_2 humified atmosphere. CAFs cultured in T-flasks were used to generate CM, and it was important to prepare the CM to a solution as pure as possible without cells and other undesired components. This was done by collecting the 8 mL of medium 48h after irradiation followed by centrifugation and then filtering of CM. Medium from T-flask was collected in 15 mL tubes and centrifuged at 350 x G for 10 minutes. Supernatant was poured in a new 15 mL tube subsequently and centrifuge again at 2000 x G for 10 minutes. CM was after the last centrifugation filtered in new 50 mL tube by using a 0.45 µL filter, and by doing so eliminating the last remaining undesired components in the solution. CM was then either used immediately for experimental purposes or frozen down at -80ºC in 50 mL tube for later assays. Collected CM was normalized in relation to number of cells in T-flask. Density of cells in T-175 flask was approximately 450.000 cells/mL. Before analyzation, CAF-CM was thawed and vortex for 30 seconds to ensure an adequate mixture of proteins in the suspension. It is important that control cells are not allowed to reach a state of high confluence, since this could result in cell death. Non-irradiated CAFs could be frozen down after generation of CM and used for later experiments, but irradiated CAFs could not be used for further studies since they become senescence after irradiation.

4.5 Isolation of PBMCs

Blood was collected from healthy donors at the Blood bank in Tromsø University hospital. Leukocyte-enriched blood (buffy coat) was centrifuge over Lymphoprep™ density gradient medium to isolate PBMCs.

4.5.1 General principles

The Lymphoprep™ method uses a density barrier as a mechanism to separate cells. Cells in the blood with a density higher than the medium (1.077 g/mL) goes through the barrier medium, such as erythrocytes and some polymorphonuclear cells. Other cells with a density lower than the medium, such as PBMCs, will remain in the interface after centrifugation

4.5.2 General procedure

Leucocyte-enriched blood was diluted 1:1 with PBS in T-75 flask to a total of 100 mL. Four 50 mL tubes was filled with 15 mL Lymphoprep™ and then 25 mL of the blood/PBS solution. This was done very carefully so the blood/PBS did not blend with the Lymphoprep™ solution but maintain as a separate solution on top of Lymphoprep™. The four tubes was then centrifuge at 800 x G for 30 minutes at 20ºC without brake or acceleration to ensure no disturbance which could disrupt the separation of blood cells. After centrifugation, PBMCs could be seen as a white layer in the middle of tube between the top (plasma) and the bottom (erythrocytes), as seen in **Figure 4.4**. The white layer was collected from all four tubes with a Pasteur pipette and filled in a new 50 mL tube and centrifuge at 350 x G for 10 minutes at 20ºC. Supernatant was removed, and pellet resuspended in 50 mL PBS and centrifuge again at 350 x G for 10 minutes at 20ºC. Supernatant were discarded after the last step of centrifugation. PBMCs were either used in experiments after isolation or frozen for later use. PBMCs destined for freezing was first counted. Freezing medium consisting of 90 % FBS and 10 % DMSO and was added to the tube with PBMCs according to how many cells that was counted. Approximately $5x10^7$ cells was filled in each CryoTube (1 mL/tube) and the cells were cryopreserved at -80ºC until further use. Before utilization of frozen PBMCs, cells were rapidly thawed in 10 mL of complete

Figure 4.4: PBMC isolation: Plasma can be seen on top and red blood cells in the bottom, while white blood cells are in the middle as white layer.

medium consisting of RPMI-1640 culture medium supplemented with 10 % FBS and 1 % penicillin-streptomycin.

4.6 Isolation of $CD14^+$ cells by immune-magnetic cell separation

Human $CD14^+$ monocytes were isolated from PBMCs with positive selection following the principle of magnetic activated cells sorting separation (MACS) (Miltenyi Biotec) by using anti-CD14-coated magnetic beads.

4.6.1 General principle

The principle of MACS separation is to isolate specific cell populations by particular surface antigens named cluster of differentiation (CD). Cells are incubated with magnetic microbeads that are coated with monoclonal antibodies against a particular CD molecule located on the surface of target cells. Solution with cells are then loaded into a MACS column and then placed in a strong magnetic field. Labelled cells with the magnetic microbeads are retained in the column as the solution passes, while other unlabeled cells in the solution passes through the column. When the column is removed from the magnetic field, cells remained attached to the column are eluted as the positively selected cell population.

4.6.2 General procedure

MACS buffer (0.2 % bovine serum albumin) was prepared by diluting MACS BSA Stock Solution 1:20 with autoMACS^{TM} Rinsing Solution. MACS buffer was then kept on ice. PBMCs isolated from buffy coats was centrifuged at 300 x G for 10 minutes, supernatant discarded and pellet (with PBMCs) resuspended in 80 μL of MACs buffer solution per 10^7 cells. After, 20 μL of CD14 microbeads per 10^7 cells was added to the solution and mixed well before incubation of solution for 15 minutes at 4ºC in refrigerator. Following incubation, cells were washed with 1-2 mL 0.2 % PBSA buffer and centrifuged at 300 x G for 10 minutes. Supernatant was discarded, and cells resuspended in 500 μL MACS buffer. Cell suspension with beads was then loaded into a MACS LS-column which was attached to a strong magnetic field. This was followed by rinsing with 3 mL of MACS buffer into the column. This step was repeated three times. Column was then removed from the magnetic field, placed on top of 15 mL tube and then 5 mL of MACS buffer was loaded to the column. A plunger was placed on top of column and pushed carefully to ensure that all microbeads with attached $CD14^+$ was removed from the column. Eluted cells was then washed by adding 10 mL of MACS buffer to tube and then centrifuged at 300 x G for 10 minutes. Supernatant was discarded, and cells resuspended in 10

mL of cell culture medium (RPMI-1640 culture medium with 10 % FBS and 1 % penicillin-streptomycin). Monocytes were then counted and labeled with CD14-FITC antibodies for analyzation with Flow cytometry.

4.6.3 DCs differentiation and maturation

Isolated monocytes was first counted with Neubauer Hematocytometry to determine number of cells. Monocytes ($6x10^6$) were suspended in 18 mL of complete medium (RPMI-1640 culture medium with 10 % FBS, 1 % penicillin-streptomycin and pyruvate) and supplemented with 100 ng/mL IL-4 and 100 ng/mL GM-CSF for differentiation of monocytes to iDCs. Monocytes were then transferred to 6-well tissue culture treated plates with $3x10^6$ cells and 3 mL of complete medium in each well and incubated at 37ºC in 5 % CO_2 humified atmosphere. After two days, a volume of 1.5 mL from each well was collected in the same 15 mL tube since all wells contained the same type of cells destined for the same purpose and centrifuged at 300 G x for 10 minutes. It is important to extract the cells carefully to avoid spontaneous activation of DCs. Supernatant was discarded and pellet with cells was resuspended in 9 mL of complete medium and 2-fold concentration of IL-4 and GM-CSF and transferred back to wells. The cells was ready to be used in assays after four more days of incubation. For maturation of mDCs, a volume of 1.5 mL is collected on day six of incubation. Cells are centrifuged at 300 x G for 10 minutes and supernatant discarded. Pellet was resuspended in 9 mL of complete medium supplemented with 25 μg/mL IL-6, 100 μg/mL IL-1β, 25 μg/mL TNF-α and 2 μg/mL PGE_2 and then transferred back to 6-well tissue culture plate incubated at 37ºC in 5 % CO_2 humified atmosphere for 24 hours. An absolute cell count of mDCs was determined by flow cytometry via light scatter signals PI fluorescence.

4.7 Co-culture of CAFs with DCs

Non-irradiated and irradiated CAFs was co-cultured with DCs to investigate the immunoregulatory effect CAFs may exert on DCs regarding cell-cell contact.

Procedure: The process of co-culturing CAFs together with DCs was divided in two parts:

- Isolation and culturing of DCs
- Isolation, culture and irradiation of CAFs

The procedure for isolation of CAFs are described in section **4.3** and the irradiation of CAFs in section **4.4**. The isolation of monocytes and differentiation into iDCs and mDCs are described

in section **4.6**. The main difference between CAF-CM and CAF co-culture assays was that CAFs needed to be cultured in 24-well tissue culture plates before irradiation when used in co-culture experiments. This is because CAFs become senescent after irradiation, which would make it difficult to detach the CAFs from flask and then make CAFs attach in plates used in co-culture with DCs. Non-irradiated and irradiated CAFs were co-cultured with DCs in a ration 1:2, respectively, in all experiments regarding co-culture of CAFs and DCs.

After 48 hours of co-culturing CAFs and DCs, DCs were centrifuge for 5 minutes at 300 x G and used in further experiments. Supernatant was transferred to new Eppendorf tube and centrifuge at 3000 x RPM for 10 minutes to exclude cell debris and frozen down at -80ºC for later use in ELISA experiments regarding the production of IL-10 and IL-12 by DCs. CAFs were attached to the 24-well tissue culture plates and this makes it possible and easy isolate the DCs after being co-cultured with CAF, since DCs are free in the solution enabling the cells for being extracted from the wells without CAFs.

4.8 Cell surface marker expression of DCs by flow cytometry

Cell surface markers represents proteins exclusively expressed on the surface of cells and are regularly used as makers of specific cell types. For characterization of monocytes, iDCs and mDCs, a panel of different surface markers was used: CD14, CD1a, CD209, CD40, CD80, CD86 and HLA-DR. It was also of interest to investigate the change in expression of surface markers when DCs were cultured with non-irradiate or irradiated CAF-CM and DCs/CAFs co-culture to compare the two conditions.

4.8.1 General principle

Flow cytometer is an instrument that can be used to analyze cells regarding size and complexity. Further, fluorochromes can be used to label specific membrane receptors and analyze receptor expression by flow cytometry. Sample solution with cells are placed inside the flow cytometer and injected into the instrument. A beam is adjusted allowing just one cell to pass through the laser at the time. When cells passes the laser, light is scattered (forward and side scatter) to a detector which gives information about the characteristics of cells passing the laser. Antibodies with attached fluorochrome has a wide fluorescence spectrum. An overlap between fluorochromes can occur when using more than one fluorochrome at the same time. This is called spectrum overlap and needs to be overcome by compensation. Each fluorochrome is

calculated as a percentage to itself and this is a mathematical process were different multiparameter data is corrected for spectral overlap. Generated data can be plotted in a single dimension which produces one- or two-dimensional dot plots based on the intensity of fluorescence emitted from analyzed cells. Regions on these plots can be separated based on the fluorescence intensity and generate a series of subset extractions. This is called gating. The dots are often made on logarithmic scales. Because different fluorescent dyes emission spectra overlap, signals at the detectors have to be compensated electronically as well as computationally.

Data analysis

Data collected from flow cytometry experiments are analyzed with FlowJo on another computer than the actual flow cytometer process. Data is organized in "workspace" which allows for a hierarchical overview of the samples and the analyzation. Samples can be organized in panels regarding antibodies or tissue/cell type.

4.8.2 General procedure

Experiments regarding surface markers included monocytes, iDCs and mDCs. Cell type used in experiments was first collected in a 1.5 mL Eppendorf tube and suspended with 100 μL MACS buffer and 2 μL of appropriate antibody (according to manufacture protocol). Solution was mixed thoroughly and incubated for 15 minutes in refrigerator (dark) at 4ºC. After the 15 minutes, cells were washed two times by adding 1-2 mL of MACS buffer and centrifuged at 1500 x RPM for 10 minutes to remove excessive antibodies in samples not bounded to cells. Pellet with cells was resuspended in 300 μL of MACS buffer and analyzed by flow cytometry. PI was used to measure the live and dead cell ratio in the experiment.

4.9 Transwell migration assay

Migration of mDCs was investigated under the influence of non-irradiated or irradiated CAF-CM and in co-culture with CAFs. This was observed in the presence or absence of chemoattractant CCL-19 in a CCR7 chemotactic-dependent mechanism using Transwell migration chambers. The final step included extraction of cells and counting with Neubauer Hematocytometry.

4.9.1 General principles

Transwell migration assay, also known as Boyden chamber, are often used to measure the movement and invasive capacity of different types of living cells towards a chemoattractant gradient. The chamber itself is comprised of a lower and an upper chamber separated by a thin membrane with pores, which allows cells of particular size to pass through (**Figure 4.5**). It is important to choose Transwell with pores that allows the target cell to pass through, but not allowing the passive migration of cells. Because of this, a membrane with smaller pores than target cells should be chosen. The chemoattractant is added to the solution in the lower chamber. After a certain time, cells in the lower chamber is collected and counted. One very important note in this experiment is that the number of cells placed in the upper chamber needs to be very accurate, since this number is used to evaluate how many cells that was able to migrate through the membrane and towards chemoattractant in lower chamber.

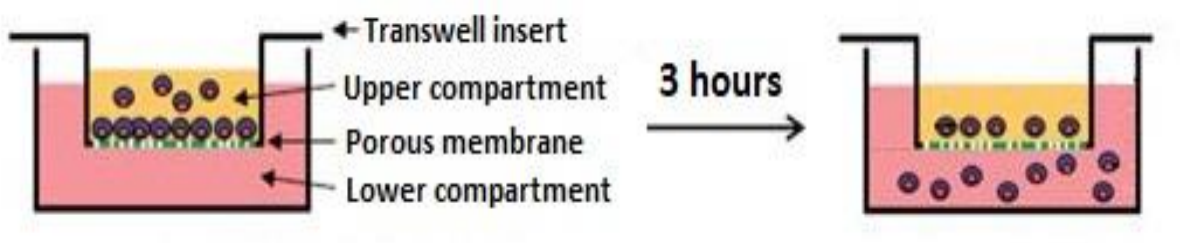

Figure 4.5: Transwell migration assay: *Cells are placed in the upper chamber (transwell insert) and was allowed to migrate towards the chemoattractant CCL19 in the lower chamber for 3 hours. Cell were afterwards counted to determine the migration capacity of cells.*

4.9.2 General procedure

mDCs were incubated with CAFs in co-culture for 48 hours before used in migration assay. Then, mDCs was collected in 50 mL tubes and centrifugated at 300 x G for 10 minutes, supernatant collected for later experimental use and cells resuspended in RPMI 1640 with 10 % FBS with a cell density of $5x10^5$/mL. The upper chamber was filled with 200 µL ($1x10^5$ cells) of RPMI solution with cells. The lower compartment was filled with 600 µL DMEM medium with 10 % FBS supplemented with Human CLL19 (MIP-3β) (Miltenyi Biotec) with a concentration at 25 ng/mL. Transwell plates with cells were then incubated at 37ºC in 5 % CO_2 humified atmosphere for 3 hours. Cells were then collected from wells and counted with Neubauer Hemocytometry.

mDCs used for CAF-CM migration experiments was also collected in 50 mL tubes and centrifugated at 300 x G for 10 minutes followed by resuspending in RPMI 1640 with 10 %

FBS to a cell density of $1x10^6$/mL. In this assay, 100 µL ($1x10^5$ cells) of RPMI solution with cells was filled in the upper chamber with 100 µL of the appropriate CAF-CM. The lower chamber was filled with 300 µL of DMEM medium with 10 % FBS, and 300 µL of correct CAF-CM supplemented with Human CLL19 (MIP-3β) (Miltenyi Biotec) with a concentration at 25 ng/mL. The remaining part of experiment was done as with co-culture migration.

4.10 Antigen uptake capacity of iDCs

The pinocytotic antigen uptake capacity of iDCs was investigated after iDCs had been under the influence of non-irradiated or radiated CAF-CM or CAFs for 48 hours. Samples with cells was analyzed with flow cytometry to measure antigen uptake.

4.10.1 General principles

One of the most important features of iDCs is the ability to take up antigens through pinocytosis, which is a form of endocytosis. Internalized antigens are then presented on the surface through MHC class II molecules. In this assay, iDCs were first cultured with CAF-CM or co-cultured with CAFs. iDCs was afterwards collected and then treated with FITC-dextran to simulated antigens. Dextran was taken up by iDCs and the FITC molecule attached to dextran can then be recognized by analyzing cells with flow cytometry to determine the fluorescent emitted by DCs/FITC dextran uptake.

4.10.2 General procedure

A certain amount of DCs ($1x10^5$ cells) were incubated with 100 µl of FITC-labeled dextran (MW 40,000 Sigma FD40S or equivalent – 1 mg/mL) in RPMI 1640 medium in 5 % CO_2 at 37ºC for 60 minutes. A control sample was kept on ice for 60 minutes to check for non-specific binding of FITC-dextran to cell surface. All samples were washed two times after 60 minutes of incubation to remove all unbound FITC-dextran in the solutions. This was done by centrifuge samples at 300 x G for 5 minutes in 4ºC with ice-cold PBS supplemented with 0.5 % BSA. Samples were kept on ice until cells were analyzed with flow cytometry. Uptake of FITC-dextran was determined by measuring the mean fluorescence intensity (MFI) of FITC by flow cytometry and dead cells was excluded from the analysis by PI fluorescence. The specific antigen uptake of FITC-dextran by iDCs was calculated by subtracting the MFI of the control sample that was kept on ice the whole time with samples incubated at 37ºC.

4.11 T cell proliferation assay by mDCs

In this assay, mDC ability to prime T cells was analyzed with flow cytometer after mDCs had being cultured with CAF-CM or in mDC/CAF co-culture. The T cell proliferation assay required both DCs and T cells. Human $CD14^+$ monocytes were isolated from PBMCs by positive selection as described in section **4.6**. Isolation of $CD4^+$ T cells from PBMCs was done by the principles of magnetic activated cells sorting separation (MACS)(Miltenyi Biotec) with Naïve $CD4^+$ T Cell Isolation Kit II, which is a negative selection. This is done by using a cocktail with biotin-conjugated monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR and CD235a. This binds all unwanted cells to microbeads and column, while target cells are eluted from the column. mDCs are able to prime the CD4 T cells when cultured together by interactions through MHC class II molecules – TCR and co-stimulatory molecules CD80/CD86 – CD28.

4.11.1 General principles

mDCs ability to prime naïve $CD4^+$ T cells were measured using CellTrace Violet solution (Life Technologies). This fluorescent dye is able to pass the cell membrane and bind to free amides inside cells. This provides a more stable and consistent signal compared to dyes binding to the cell membrane. CellTrace Violet acts without affecting the cells biology or proliferation. Cells of interest are incubated with dye, which enters cells and fluorescent intensity is measured. As the cells divides (proliferates), the dye becomes "diluted" amongst daughter cells, which results in less and less dye inside cells after each stage of cell division. Then, cells are analyzed again after a certain time with flow cytometry to measure the fluorescence intensity indicating proliferation.

4.11.2 General procedure

The isolation of $CD14^+$ with positive selection and differentiation into iDCs and mDCs are described in section **4.6**. The isolation of $CD4^+$ T Cells from PBMCs started with preparation of MACS buffer containing phosphate-buffer saline (PBS), pH 7.2, 0.5 % bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing solution. PBMCs used for isolation of $CD4^+$ T Cells was then centrifugated at 300 x G for 10 minutes, supernatant discarded and cells resuspended in 40 μL of MACS buffer per 10^7 of total cells. Then, 10 μL of Naïve $CD4^+$ T cell Biotin-Antibody Cocktail II per 10^7 cells was added and solution was mixed thoroughly and incubated for 5 minutes in refrigerator (2-8ºC). Another 30 μL of MACS buffer per 10^7 cells was added followed by 20 μL of Naïve $CD4^+$ T

Cell MicroBead Cocktail II per 10^7 of total cells. Solution was well mixed and incubated in refrigerator (2-8ºC). Solution was then loaded to a MACS LS column attached to a strong magnetic field. Colum was first prepared by rinsing the column with 3 mL of MACS buffer. Cell suspension was then loaded in the LS column. Unwanted cells will bound to the magnetic column, while the enriched naïve $CD4^+$ T cells will be in the flow-through that was collected. Column was washed by adding 3 mL of MACS buffer to the column to ensure removal of all enriched naïve $CD4^+$ T cells from column.

The purity of isolated enriched naïve $CD4^+$ T cells was determined by labeling cells with i) CD4-PE or ii) CD45RO-APC and CD45RA-PE antibodies (Miltenyi Biotec) followed by analyzation with flow cytometry using MACSQuant Analyzer 10. Then, a total of $5x10^6$ purified naïve $CD4^+$ T cells was suspended in 400 µL PBS and labeled with 100 µL of a 10 µM CellTrace Violet solution (Life Technologies) for 5 minutes at room temperature. Cells were afterwards washed one time with 1 mL of ice-cold PBS supplemented with 1 % fetal calf serum and then three times with 2 mL MLR medium (RPMI 1640, 2 mM L-glutamine, non-essential amino acids, 0.1 mM sodium pyruvate, 5 % human antibody serum). In the last step, naïve $CD4^+$ T cells were resuspended in MLR medium with a cell density of $5x10^5$ cells/mL. mDCs were suspended in MLR medium as well at a cell density of $1x10^6$ cells/mL and diluted 1:2. The different dilutions of cells were transferred to 96 well plate with 100 µL of diluted solution in each well. Then, solution with naïve $CD4^+$ T cells were added to wells with 100 µL of solution in each well. Plates with cells were incubated for 7 days at 37ºC, 5 % CO_2. The proliferation of $CD4^+$ T cells was determined by measuring dilution of cell-associated CellTrace Violet by flow cytometry. Dead cells and debris was excluded from the analysis by scatter signals and PI fluorescence. As the T cells proliferate, the intensity of fluorescence becomes lower.

4.12 Enzyme linked immunosorbent assays (ELISA)

Enzyme linked immunosorbent assay (ELISA) was done to quantify the production of IL-10 and IL-12 by mDCs.

4.12.1 General principle

The sandwich ELISA is a method used to quantify antigens between two layers of different antibodies (i.e. capture and detection antibody). Antigen in sample needs to contain a minimum of two different antigenic epitopes, which are capable to bind antibody, because at least two antibodies acts in the sandwich. Either monoclonal or polyclonal antibodies are used in Sandwich ELISA as capture and detection antibodies. Monoclonal antibodies can recognize one single epitope which allows accurate detection and quantification of small differences in antigen. A polyclonal is most often used as the capture antibody to pull down as much of the antigen as possible, and the capturing antigen is attached to the surface of the 96-well plate used in ELISA. Streptavidin is used to detect antigens and binds to detector antibodies that conjugates with an enzyme named Horseradish Peroxidase (HRP) which is detected by the substrate Tetramethylbenzidine (TMB). The enzyme – substrate complex converts the substrate to a colored product in the reaction, which means that ELISAs uses a colorimetric substrate as a reporter for detection. One advantage of using Sandwich ELISA is that the sample does not need to be purified before the analysis. The method is very sensitive and can be 2 to 5 times more sensitive compared to direct or indirect ELISA. The principle behind Sandwich ELISA can be seen in **Figure 4.6**.

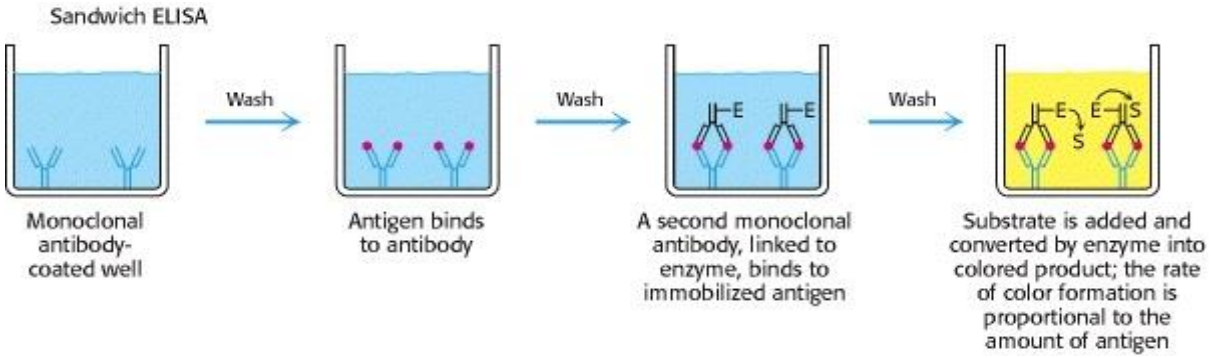

Figure 4.6: Principle of Sandwich ELISA: *Plate with wells are coated with capture antibody. Wells are washed before samples are added, which binds to capture antibodies. The wells are then washed again followed by adding of detection antibodies. Wells are washed to remove all unbound antibodies and HRP-conjugated streptavidin is pipetted to wells resulting in a colorimetric substrate solution.*

4.12.2 General procedure

A total of four ELISAs was done in the study to determine the concentration of IL-10 and IL-12 produced by mDCs in the presence of CAF-CM or in mDC/CAF co-culture. Concentrations of IL-10 and IL-12 was determined from supernatant obtained from experiments involving mDCs cultured for 48 hours with either CAF-CM or in co-culture with CAFs. The concentration was determined by using Human IL-10 DuoSet® and Human IL-12 DuoSet® ELISA kits. The sample for IL-12 detection was diluted 1:4 with Reagent dilution, while sample for IL-10 was not diluted. Kits were used in duplicates as according to product protocol. Reading of plates used in ELISA was recorded through a Multiskan Ascent microplate reader at a wavelength of 450 nm. Ascent Software was used for the construction of standard curves and for the determination of protein concentrations in the different samples. Curve fitting was done as described by the manufacture instruction with a four – parameter logistic algorithm.

All reagents and solutions used in the experiment was purchased individually and reconstituted according to the instructions. Standard curves were established by two-fold serial dilutions and run in triplicates in all ELISA test. Blank was also in triplicates. The experimental control contained iDCs and mDCs.

4.13 Statistical analysis

The results from all experiments represents the average measurements from all donors with standard deviations (S.D) and displayed as a fold of DCs control. Regarding the experiments with ELISA, only readings above the detection limit of the assays are included in the results. Data from experiments was analyzed using paired Student`s T-test. And P values < 0.05 was regarded as statistically significant. Graph pad prism 7.00 was used for analyzation and Microsoft Excel 2018 and Graph pad prism 7.00 for creating graphs.

5 Results

5.1 Isolation of primary cancer-associated fibroblasts and radiation protocols

CAFs were isolated from specimens resected from patients with non-small-cell lung carcinoma. The purity of isolated and culture expanded cells was determined by linage-specific markers including α–SMA and FAP-1 (not shown). Isolation of CAFs was followed by irradiation with clinical linear accelerator according to protocols established by Hellevik et al [168] to generate CAF-CM and CAFs for use in co-culture experiments with DCs. **Figure 5.1** represents CAFs morphology before and after irradiated with 3x6 Gy and 1x18 Gy. CAFs acquired a flat and enlarge morphology after irradiation which could be an indicative of cell growth arrest.

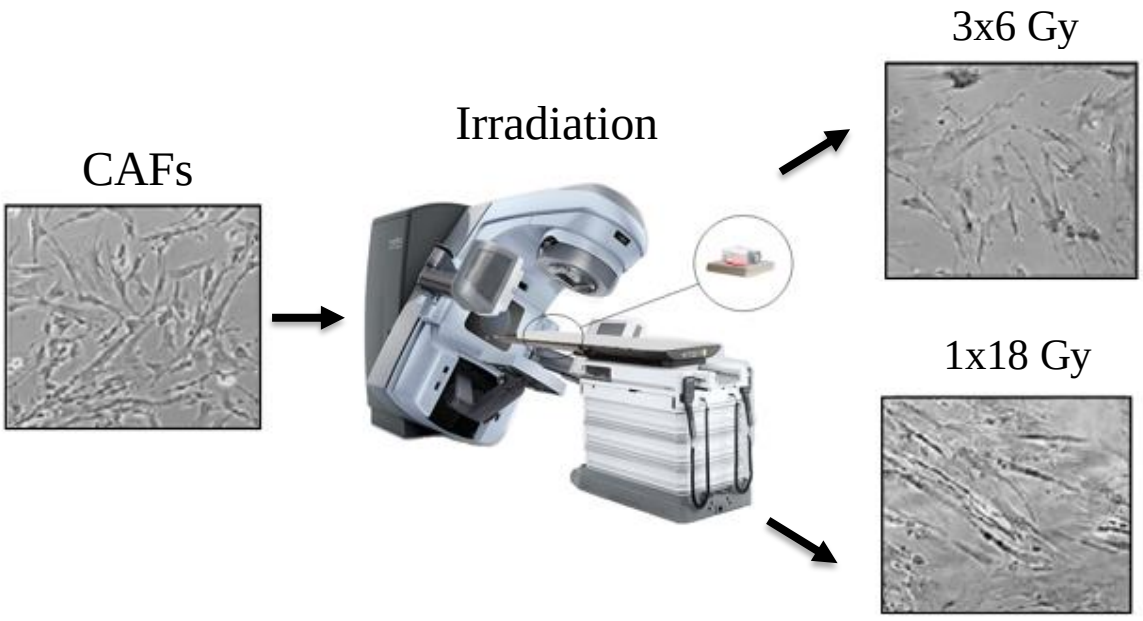

Figure 5.1: Irradiation of cancer-associated fibroblasts: *CAFs isolated from patients with NCSLS and culture-expanded for 3 passages were exposed to two different regiments of irradiation: medium-dose fractioned irradiation (3x6 Gy) and single high-dose irradiation (1x18 Gy) to produce both CAF-CM and CAFs used for various experimental settings.*

5.2 Isolation of CD14$^+$ monocytes and differentiation into immature and mature DCs

The result from isolation of monocytes and generation of DCs can be seen in **Figure 5.2**. Isolated monocytes can be observed with an irregular morphology with a relatively small cell size. Differentiated iDCs was seen with much larger cells size compared to monocytes and had more distinct spherical morphology. The following maturation of iDCs generated mDCs with dendritic structures.

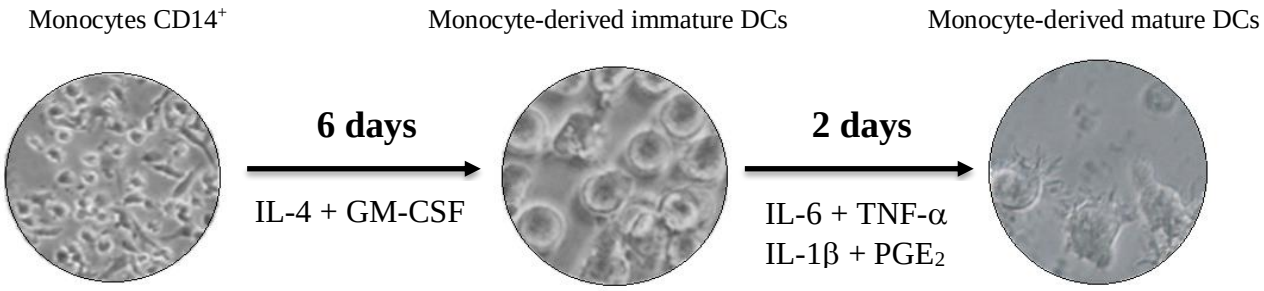

Figure 5.2: Generation of monocytes, iDCs and mDCs: *Monocytes were isolated from PBMCs and differentiated into iDCs by culturing monocytes with cocktail containing GM-CSF and IL-4. For generation of mDCs, iDCs were incubated for 48 hours with a cytokine cocktail comprising IL-6, TNF-α, PGE_2 and IL-1β.*

5.3 Gating strategy for flow cytometry analyses

DCs transformation was checked by surface expression of known DC differentiation markers. An array of surface markers was used, which included CD14, CD1a, CD209, CD80, CD86, CD40 and HLA-DR (MHC class II). For determination of different cell populations and the assortment of antibodies used as surface markers, it was important to use the correct gating when applying samples to flow cytometry. The gating strategy can be seen in **Figure 5.3**. A) After isolation of CD14$^+$ cells by antibody-coated magnetic beads from the total pull of PBMCs, the population of DCs was selected based on the forward scatter (size) and side scatter (complexity), B) Single populations of cells was selected C) All CD14$^-$ cells were excluded, D) For monocytes, cells of interest expressed CD1a^{high}/CD209^{high} and target cells as can be seen in the upper right corner, E) iDCs express lower amount of HLA-DR and CD80 indicated with red, compared to mDCs with high expression of HLA-DR and CD80 indicated with blue.

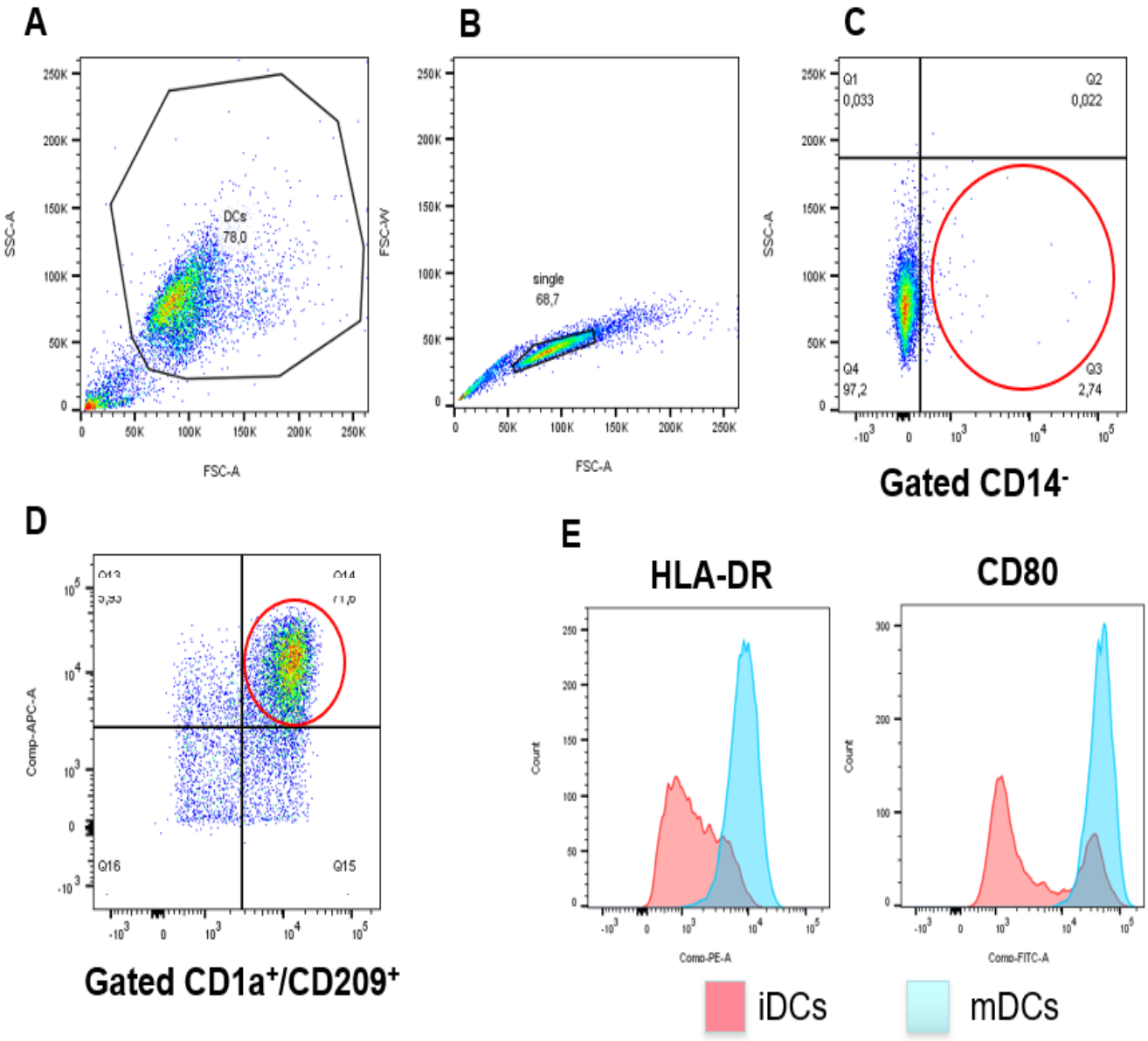

Figure 5.3: Gating strategy: A) *The population of DCs were gated and can be observed as large cells compared to lymphocytes and monocytes,* ***B)*** *Single population of cells was selected while cluster of cells was excluded,* ***C)*** *All CD14^{-} cells was excluded,* ***D)*** *Cells of interest expressed CD1a^{high}/CD209^{high},* ***E)*** *iDCs can be identified by their low expression of HLA-DR and CD80 compared to mDCs with high expression.*

Specific antibodies were used to detect the variety of surface markers included in the study. Unstained cells were used to establish cut-off of cellular autofluorescence, and each of the antibodies was gated. **Figure 5.4** is an example of the gating strategy for CD14, CD1a and CD209. The expression of CD14 amongst iDCs are 72.5 %, as compared to 2.75 % for mDCs who have lost almost all expression of CD14. The expression of CD1a is higher for iDCs with 72.5 %, while expression is 58 % for mDCs. The same is the case for surface marker CD209 were iDCs expression is measured to 72.8 % compared to mDCs with 53.3 %.

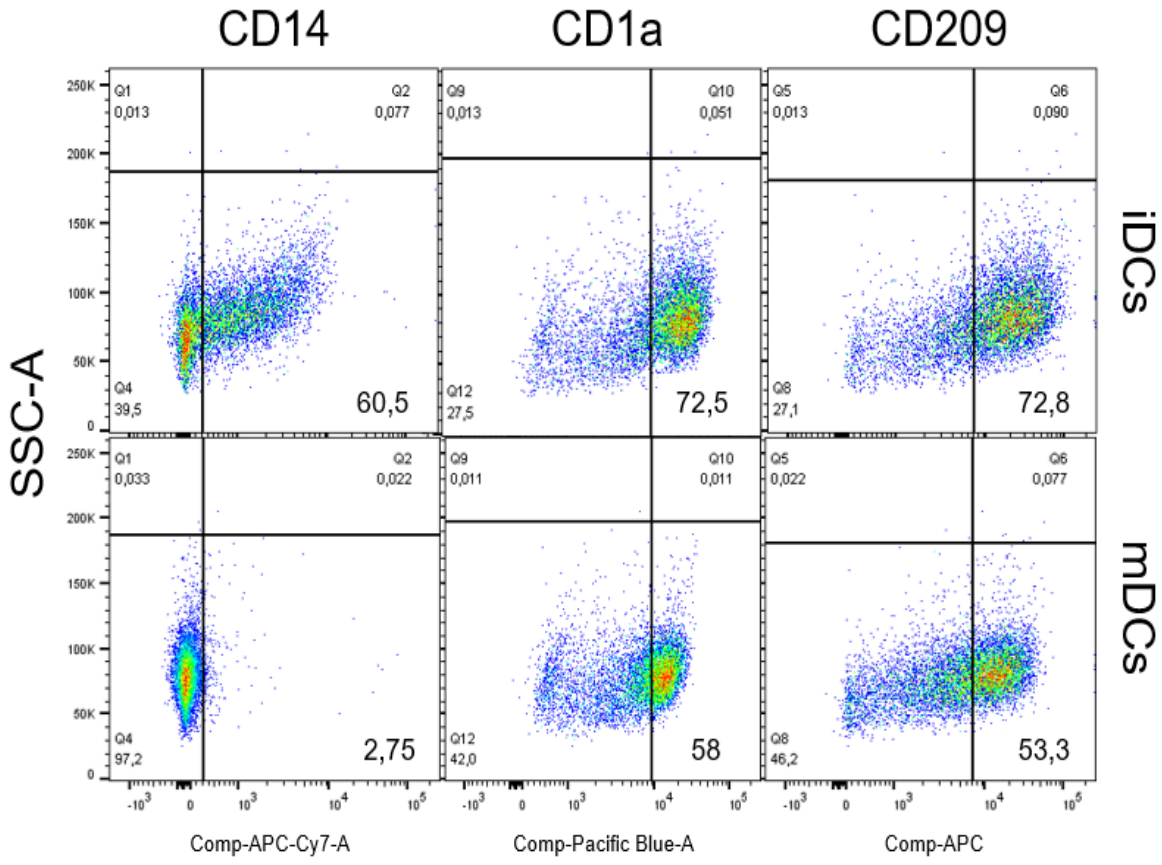

Figure 5.4: Gating strategy: *iDCs and mDCs generated from monocytes were incubated with antibodies specific for CD14, CD1a and CD209 followed by analysis with flow cytometry. This provided information about level of expression of the different markers, helping to identify different phenotypes of DCs. This was later used to observe if CAFs in either CAF-CM or co-culture experiments modulate the expression of surface markers and thus the phenotype of DCs.*

5.4 CAFs regulate monocyte-to-dendritic cell transdifferentiation

First, we wanted to investigate the expression of surface markers related to the differentiation from monocytes to iDCs, and if this transition was influenced by CAFs. It was also an objective to rule out if CAF-mediated effects were dependent on paracrine signaling or cell-cell interactions and if irradiation induced any alterations on CAFs modulating affect. We could first observe from the result in **Figure 5.5** that cytokine cocktail was working as expected, since monocyte marker CD14 was downregulated in transformed DCs, whereas iDCs markers CD1a and CD209 were elevated. Further, CAFs (both CM and co-cultures) were able to block phenotypic transformation of monocytes. DCs cultured with non-irradiated CAFs in co-culture showed increased expression of CD14 (almost 50%), reduced 80 % of the expression of functional marker CD1a and around 45 % for CD209, compared with controls. However, CAFs induce immunoregulating effect on the expression of surface markers varies. The strongest effects on DCs transdifferentiation were achieved during co-culture conditions, those suggesting that at least some of the CAF-mediated effects are exerted via cell-cell interactions. Irradiation neither enhance or suppress expression when comparing with non-irradiated CAFs, except CD1a expression in CAF-CM experiments. CAFs irradiated 3x6 Gy lose to some extent the blocking effects observed with non-irradiated CAFs.

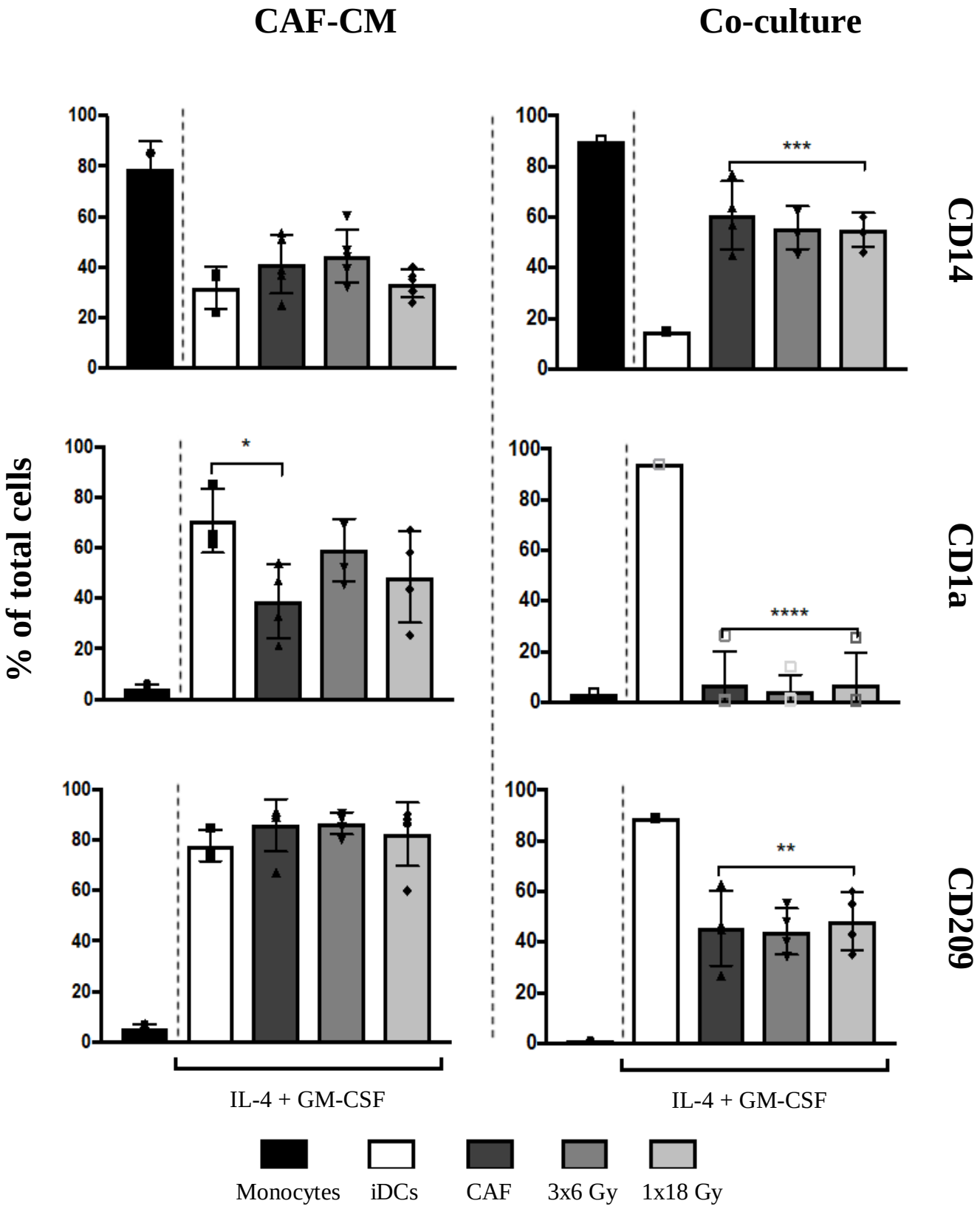

Figure 5.5: Monocytes to iDCs differentiation markers (CD14, CD1a and CD209): *Monocytes were cultured with cocktail containing IL-4 + GM-CSF for differentiation into iDCs. In addition, non-irradiated and irradiated CAFs was used to investigate CAFs immunoregulatory role in the differentiation. Expression of surface markers was analyzed with flow cytometer and results represents mean value obtained from four different CAF donors. Data were calculated by using Student`s T-test and p-value (*<0.05) was calculated between controls and non-irradiated and irradiated CAFs.*

5.5 CAFs regulate immature-to-mature dendritic cell differentiation

CAFs effects were further investigated with the same surface markers in experiments regarding maturation of DCs. The results from these experiments are shown in **Figure 5.6**, and the data suggest that CAFs could utilize paracrine signaling and direct cell-cell contact communication for their control of DCs maturation. The experimental data indicate that CAFs hinder DCs maturation as the expression levels of CD1a are comparable to iDCs (increased 10 % for CAF-CM and 15 % for co-culture). Radiation exposure was able to partly revert CAF-mediated effects on CD14 expression in experiments with CAF-CM. Whilst CAF-CM modulates an upregulation of the expression of CD14 with approximately 15 %, irradiation of CAFs with both 3x6 Gy and 1x18 Gy reverted the modulating effect of CAFs and mDCs express the same level of CD14 as control with a significant result ($p=<0.05$). Same tendencies were observed in co-culture conditions but without reaching significance.

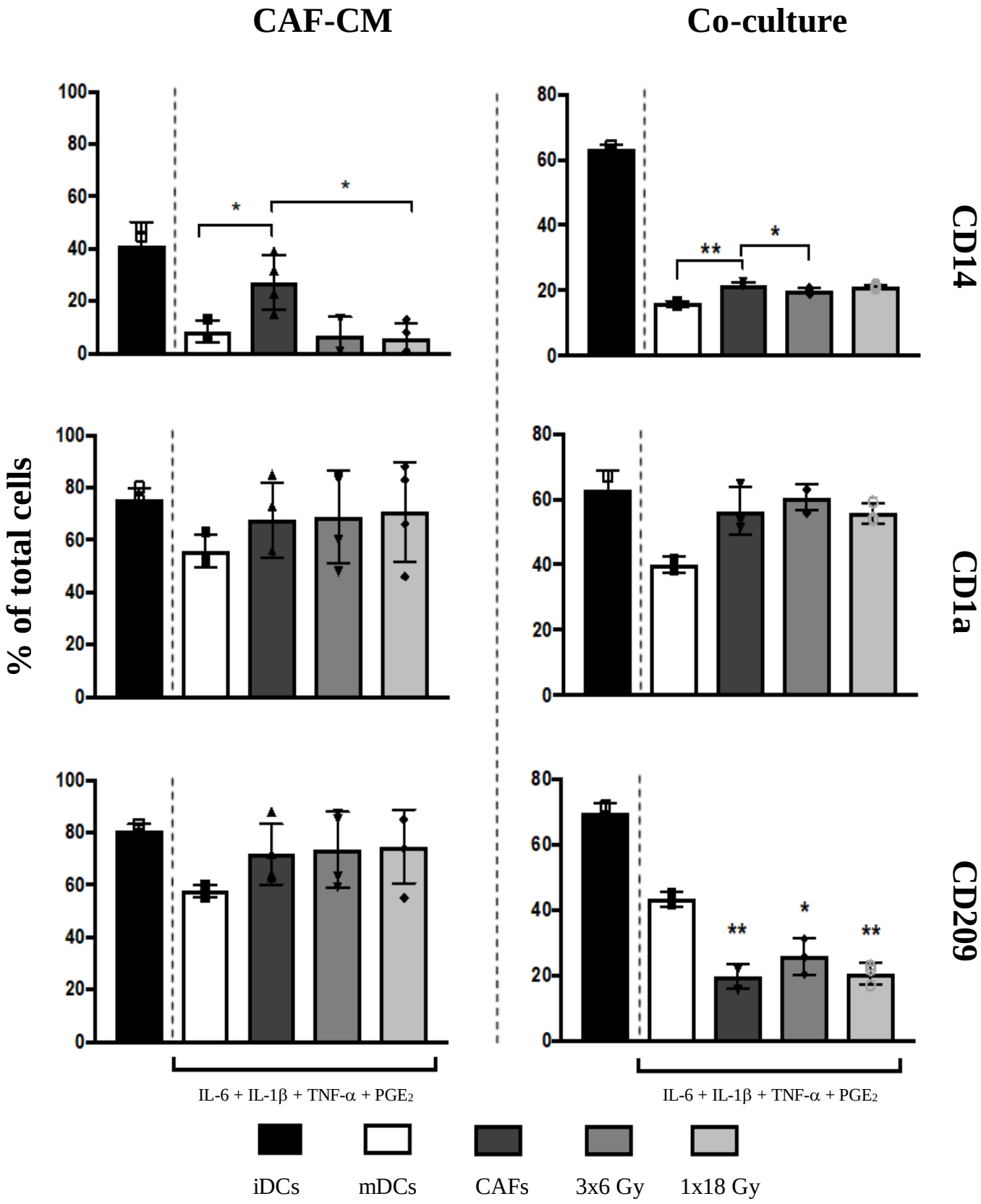

Figure 5.6: iDCs into mDCs differentiation markers (CD14, CD1a and CD209): *iDCs were incubated with cocktail containing IL-6 + IL-1β + TNF-α + PGE_2 for maturation into mDCs. Non-irradiated and irradiated CAFs were used to observe CAFs modulation regarding maturation. Expression of surface markers was evaluated with flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated with Student`s T-test and p-value (*<0.05) was calculated between controls and non-irradiated and irradiated CAFs.*

5.6 CAFs affect expression of maturation markers CD80 and CD86

The expression of co-stimulatory receptors normally expressed in mature DCs was studied. Here, we used surface markers CD80, CD86, CD40 and HLA-DR, and CAFs modulating effect on the expression of these surface markers can be seen in **Figure 5.7**. We could observe that CAF-CM exerted stronger suppressive effects on the expression of CD80 and CD86 compared co-culture conditions. Fractioned radiation reverted the immunoregulatory effect of CAFs supernatants with significant result ($p<0.05$). Very small differences in the expression of CD80 and CD80 could be observed when DCs were co-cultured with irradiated and non-irradiated CAFs, Co-cultures with irradiated CAFs resulted in enhanced expression of CD80 by DCs with statistically significant result ($p<0.05$).

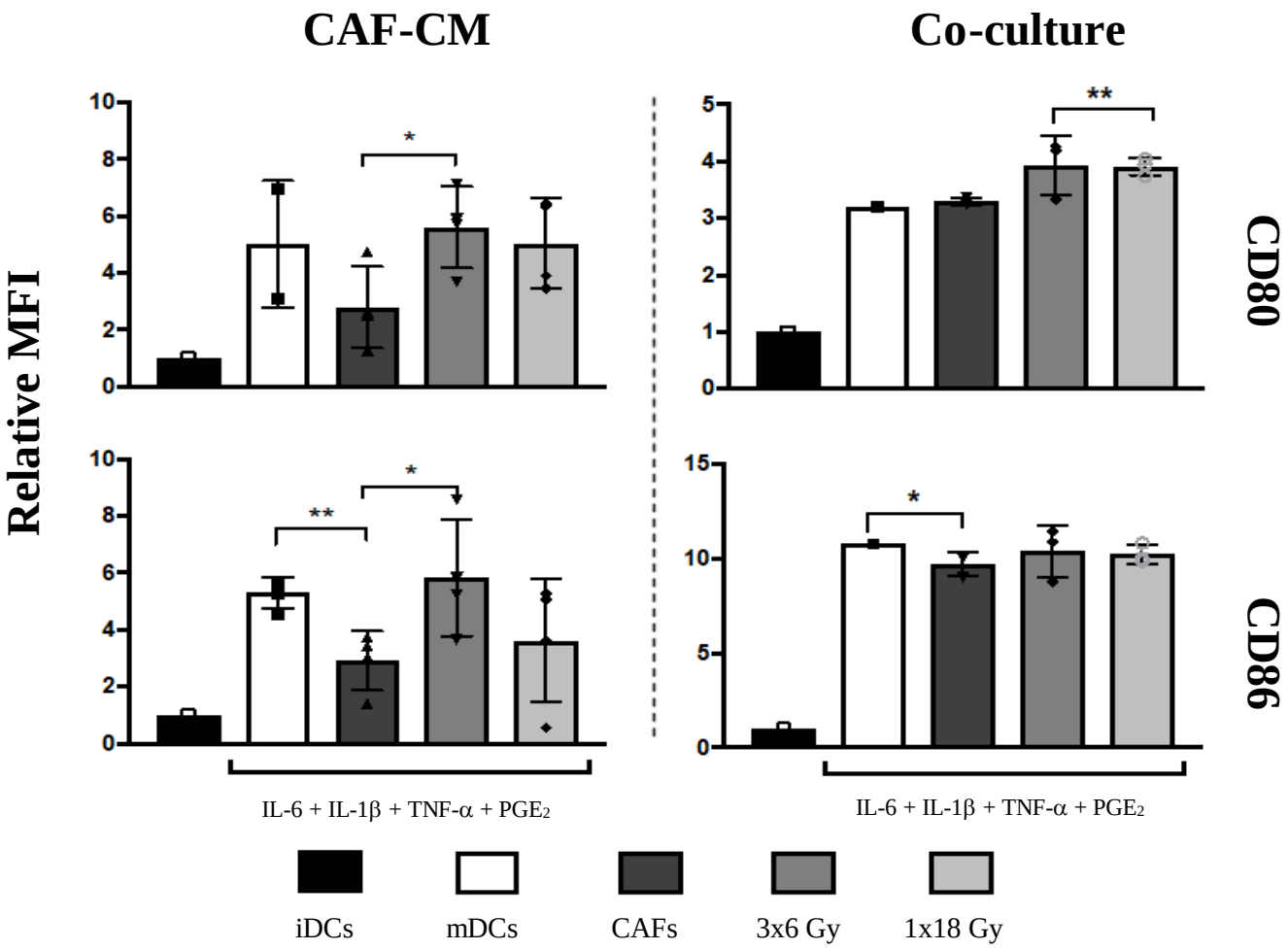

Figure 5.7: iDCs into mDCs maturation markers (CD80 and CD86): *iDCs were cultured with cocktail containing IL-6 + IL-1β + TNF-α + PGE_2 for maturation into mDCs. Furthermore, non-irradiated and irradiated CAFs were used to investigate CAFs immunomodulating function regarding functional marker expression. The expression of surface markers was analyzed with flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated with Student`s T-test (p-value = *<0.05). Result is presented as mean fluorescence intensity (MFI).*

5.7 CAFs regulate expression of CD40 and HLA-DR

We also wanted to investigate the possible modulation of surface markers CD40 and HLA-DR. The data from experiments can be seen in **Figure 5.8** and indicates that both CAF-CM and CAFs in co-culture with DCs are capable to induce changes in the expression of maturation markers. Same tendencies can be seen in all conditions, were CAFs blocks expression of both CD40 and HLA-DR through paracrine signaling and possible cell-cell mediated interactions, resulting in education of mDCs towards iDCs phenotype. Fractioned radiation hinder CAF-mediated immunosuppressive effects on DCs maturation, whereas CAF irradiated at a single high-dose behave in a similar was as non-irradiated CAFs.

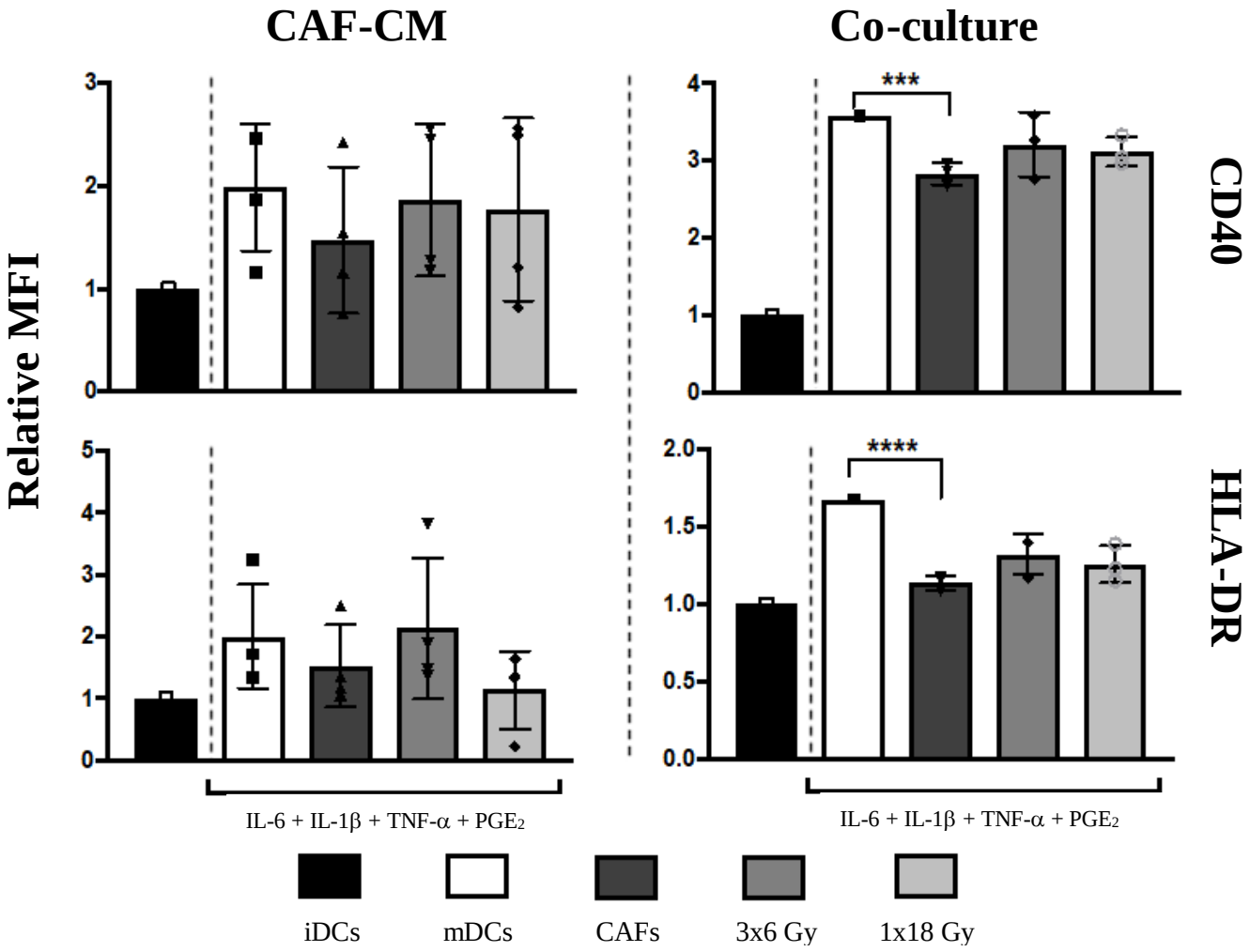

Figure 5.8: iDCs into mDCs maturation markers (CD40 and HLA-DR): *iDCs were cultured with cocktail IL-6 + IL-1β + TNF-α + PGE_2 to stimulate DCs maturation. In addition, non-irradiated and irradiated CAFs were used to investigate CAF immunoregulatory role in the maturation. Expression of functional markers was analyzed with flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated with Student`s T-test (p-value = *<0.05) and result is presented as mean fluorescence intensity (MFI).*

5.8 CAFs modulate DCs immune functions

In previous experiments, the focus was put on phenotypic markers that are used to distinguish the maturation state of DCs. These results demonstrated that CAFs may use paracrine signaling and cell-cell interactions to influence DCs phenotype. We followed these experiments by analyzing CAF-effects on DCs function. For this, we used three hallmark functions associated to DCs; antigen uptake, cell migration and priming of naïve $CD4^+$ cells.

5.8.1 Antigen uptake capacity in iDCs is suppressed by CAFs

The result from antigen uptake can be seen in **Figure 5.9** and illustrates uptake of FITC-dextran over a time period of 60 minutes. As expected, controls demonstrate that mDCs display low uptake of dextran compared to iDCs. Data indicates that non-irradiated CAF-CM blocks antigen uptake for iDCs, and the effect is reverted with fractioned irradiation of CAFs but not with single high-dose. CAFs in co-culture has marginal suppressive function on antigen uptake by iDCs, and irradiation of CAFs enhance the antigen uptake of iDCs, but not with a significant result.

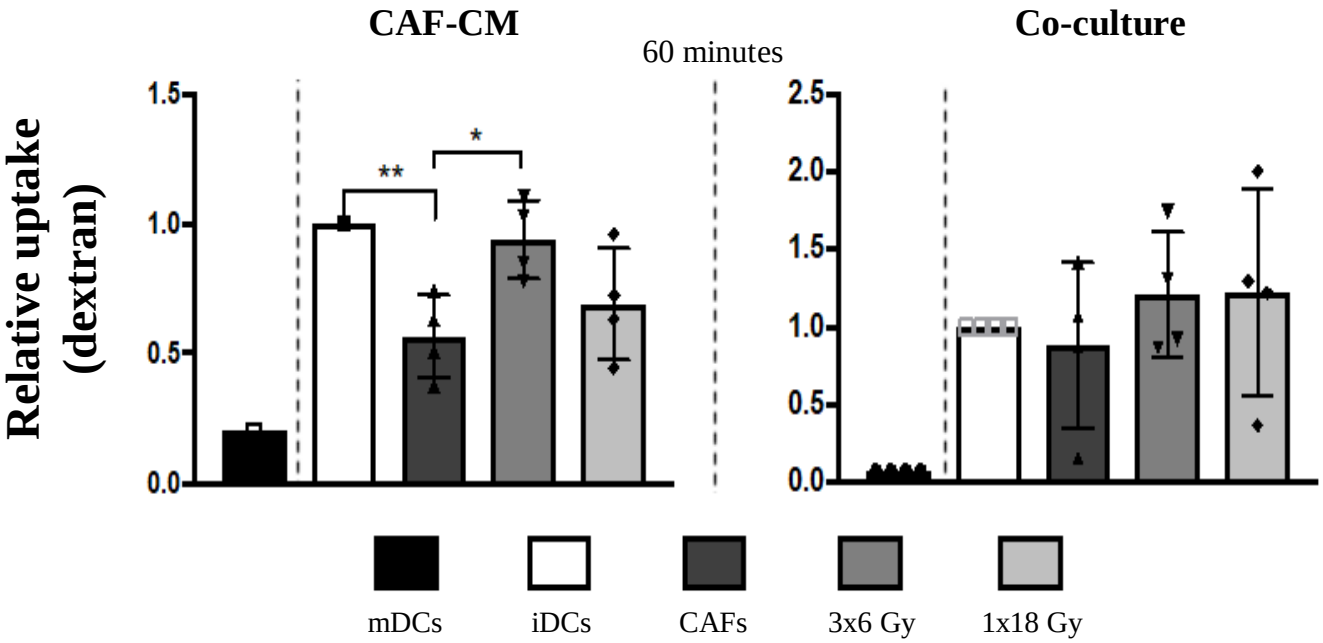

Figure 5.9: Antigen uptake of iDCs: *Untreated mDCs and iDCs were used as controls in antigen uptake for comparison. Further, iDCs were cultured with non-irradiated and irradiated CAFs to investigate CAFs modulation of iDCs uptake of FITC-dextran. The result was collected by flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated by using Student`s T-test and p-value (*p<0.05) was calculated between controls and non-irradiated and irradiated CAFs. Results are presented as fold M0 controls.*

5.8.2 DCs migration capacity is reduced by CAFs

Result from migration assay can be seen in **Figure 5.10,** and controls indicates that iDCs have low migratory capacity compare to mDCs as expected. But when mDCs have been subjected to CAF-CM or CAFs themselves before used in the migration assay, the migratory effects (almost 50%) have been lost. Fractioned irradiation of CAFs curtails the effect observed by non-irradiated CAF-CM on the migration capacity of mDCs, but not if CAFs were irradiated with single high-dose irradiation. However, in co-culture experiments radiation of CAFs blocks the migration of mDCs stronger than non-irradiated CAFs (p=<0.05).

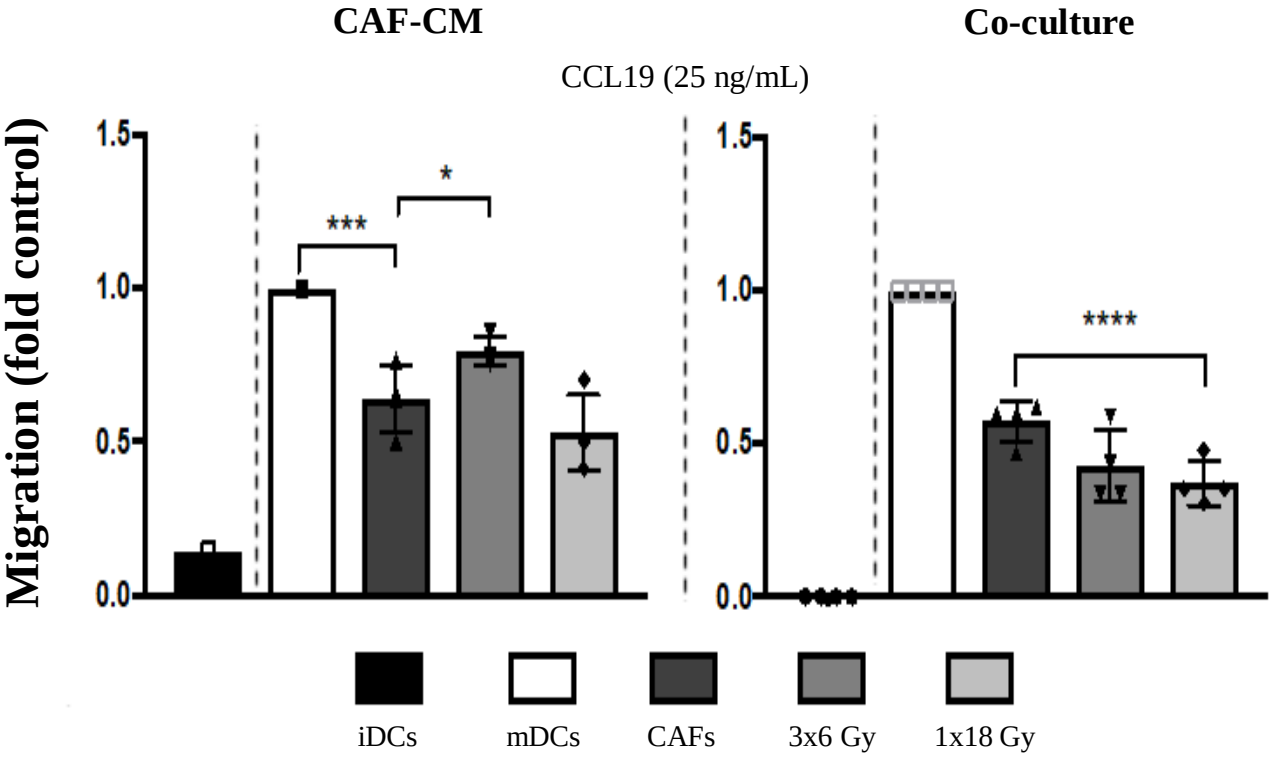

Figure 5.10: Effects of CAFs on the migration capacity of mDCs: *iDCs and mDCs was used in control to compare migration capacity. Further, mDCs was cultured with non-irradiated and irradiated CAFs to investigate CAFs immunoregulatory function regarding mDCs migration towards chemoattractant CCL19. Migrated cells were counted, and results represents mean value obtained from four different CAF donors. For analyses, pair comparisons were done among all experimental groups. Results are presented as fold M0 controls.*

5.8.3 CAFs reduces mDCs priming capacity of naïve CD4$^+$ T cells

The next step in our investigation of CAFs immunoregulatory role included mDCs priming of naïve CD4$^+$ T cells, causing proliferation of T cells. For the proliferating experiments, isolated CD4$^+$ T cells were incubated with mDCs in a ration 2:1, respectively. To measure the proliferation, T cells were labeled with CellTrace CFSE. Results are shown in **Figure 5.11** and controls demonstrate minimal T cell priming by iDCs while mDCs display high priming capability. The experiments indicates that DCs ability to prime CD4 T cells is suppressed in CAF-CM and co-culture experiments. A loss of approximately 10 % of priming ability for mDCs can be seen with CAF-CM, and for experiments regarding co-culture experiments the number is approximately 20 %. The result also demonstrated that CAFs subjected to either medium-dose fractioned (3x6 Gy) or single high-dose (1x18 Gy) did not affect CAFs modulating function of T cell priming significantly in any of the experimental settings (p=<0.05)

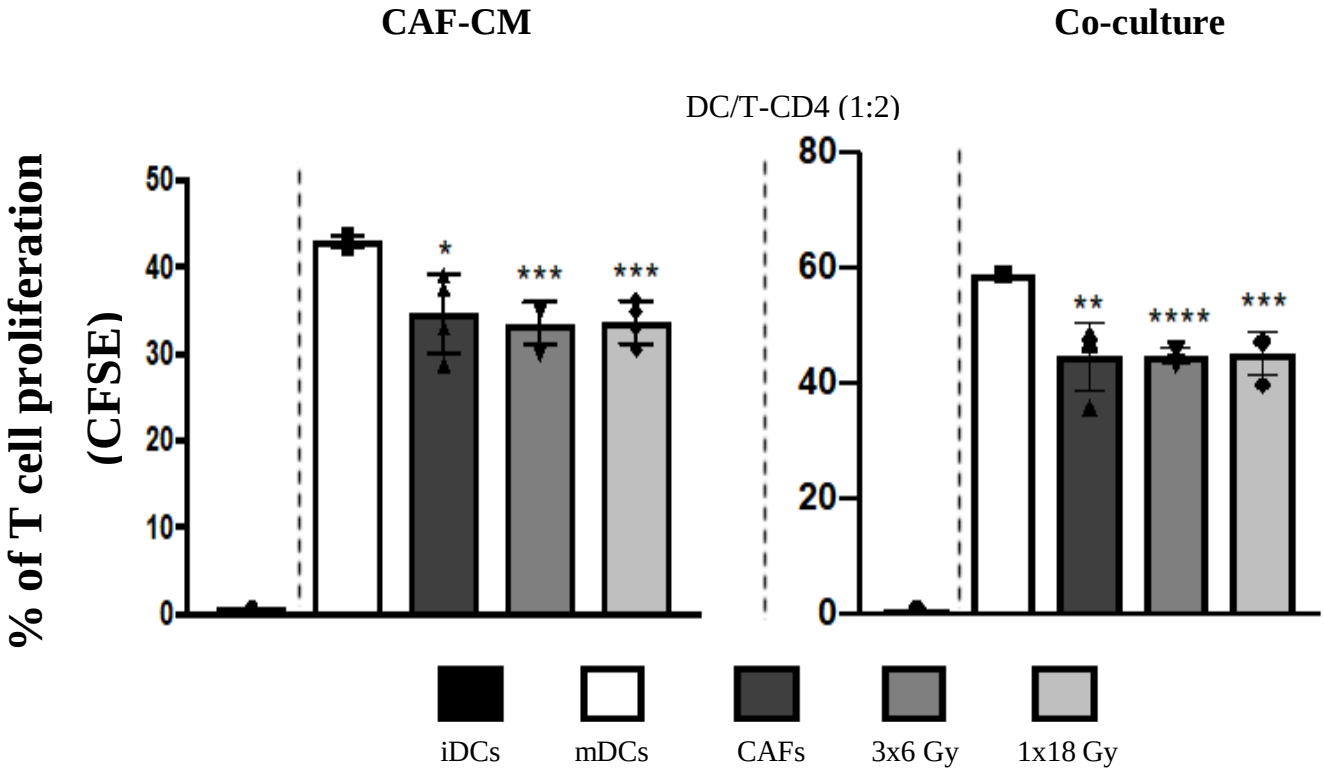

Figure 5.11: Irradiated and non-irradiated CAFs equally reduce the T cell priming abilities of matured DCs: *Untreated iDCs and mDCs was used as control for priming of naïve CD4$^+$ T cells for comparison. mDCs was in addition cultured with non-irradiated and irradiated CAFs to observe CAFs modulation of mDCs priming ability. The results was evaluated with flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated with Student`s T-test and p-value (*<0.05) was calculated between controls and non-irradiated and irradiated CAFs. Results are presented as fold M0 control.*

5.9 Cytokine production by DCs is affected by CAFs

We continued our investigation of CAFs immunosuppressive role towards DCs by determination of cytokine profile from DCs under the influence of CAFs. The result from IL-12 measurements with ELISA can be seen in **Figure 5.12**. and the experiment with CAF-CM demonstrated that non-irradiated CAFs blocks the production of IL-12 by DCs, while irradiation of CAFs reverted the modulating function of CAFs to some extent. On the contrary, experiments with DCs in co-culture with CAFs resulted in an increase of DCs production of IL-12. This production was further enhanced when CAFs was irradiated with 3x6 Gy and 1x18 Gy.

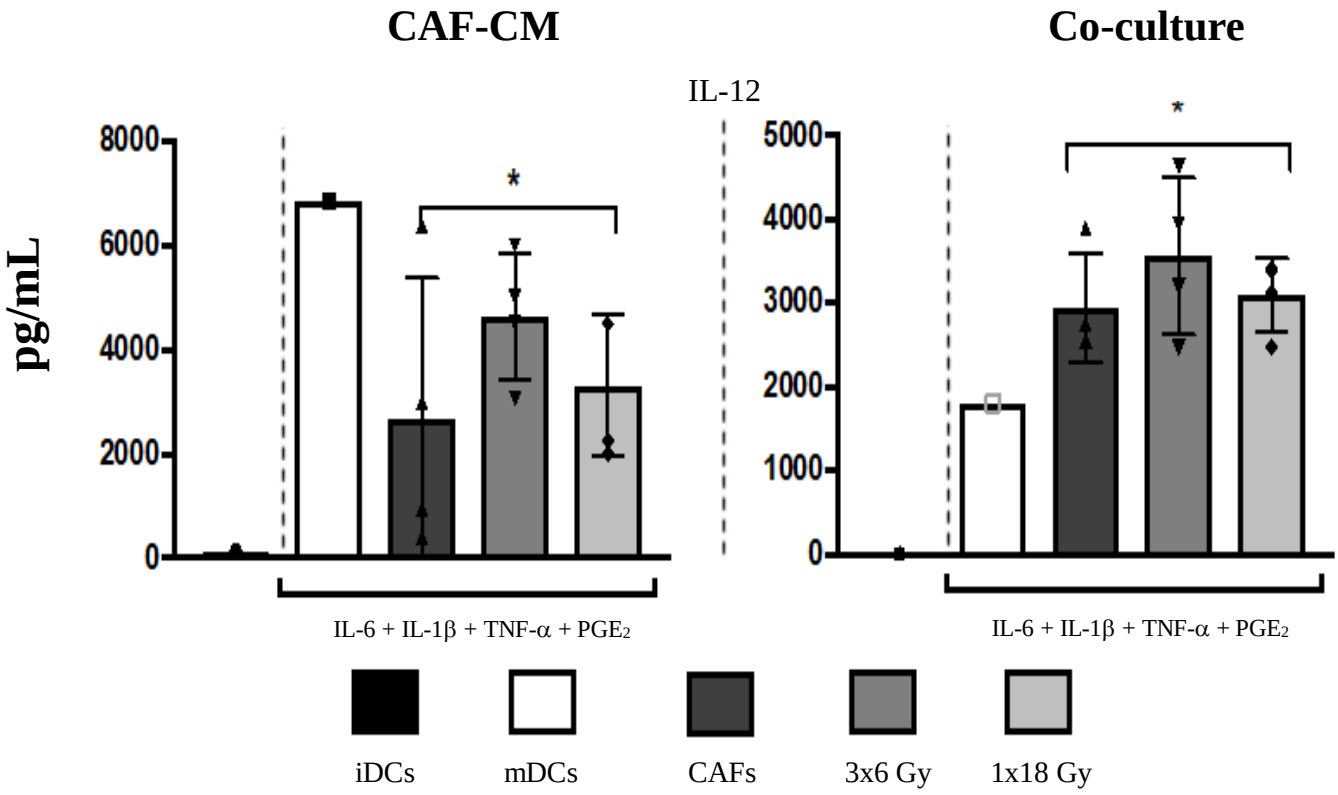

Figure 5.12: IL-12 production by mDCs is affected by CAFs: *Supernatant collected from co-culture experiments with non-irradiated and irradiated CAFs were used to measure production of IL-12 by mDCs. In addition, iDCs supernatant was used as control for comparison. Results were analyzed with flow cytometry and results represents mean value obtained from four different CAF donors. Data was calculated by Student`s T-test and p-value (*<0.05) was calculated between controls and non-irradiated CAFs. Results are presented as fold M0 controls.*

We also wanted to investigate IL-10 production by DCs under the influence of CAFs. The result can be seen in **Figure 5.13**, and the data indicates low concentration of IL-10 produced by DC under normal conditions. Our data suggest again two very different scenarios for CAF-CM and co-culture experiments. Mature DCs express lower levels of IL-10 than immature DCs. Conditioned medium from all CAF groups have no effect on IL-10 secretion. On the other hand, in co-culture settings, irradiated and non-irradiated CAFs promote IL-10 secretion by mDCs It has to be taken in consideration that measured level of IL-10 with Elisa was generally very low.

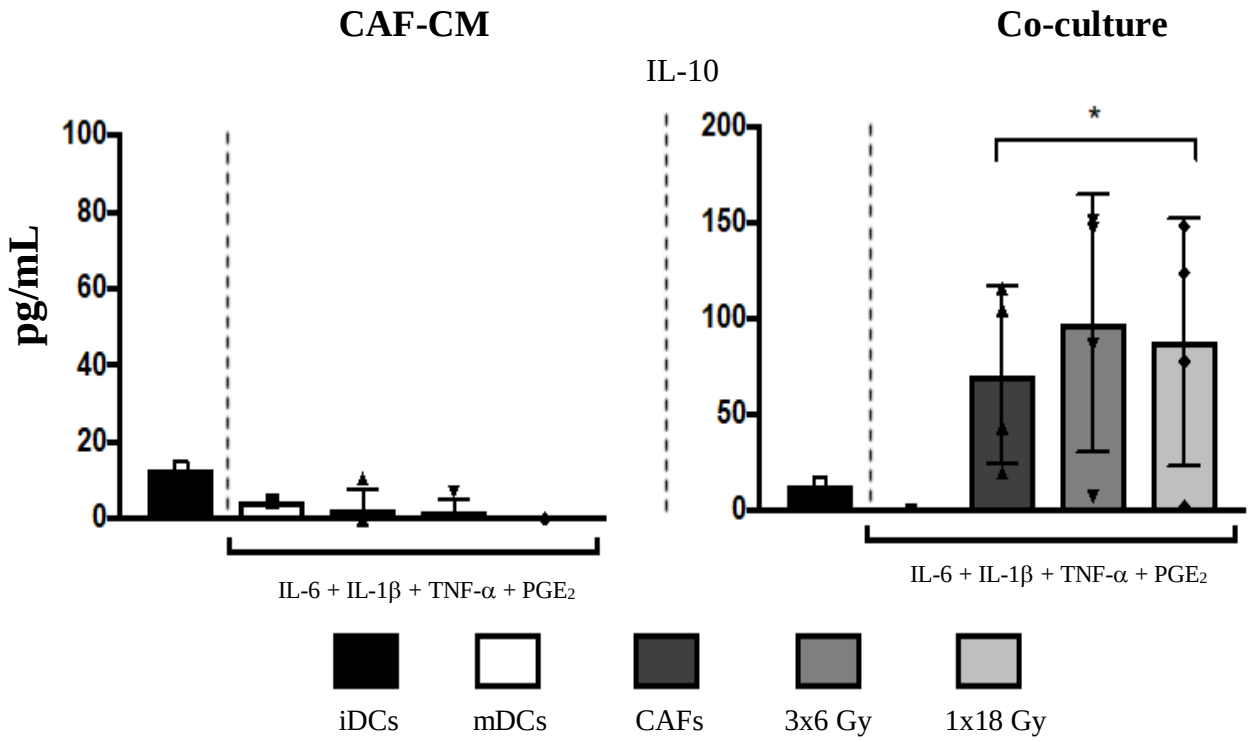

Figure 5.13: CAFs regulate production of IL-10 by mDCs: *Supernatant gathered from co-culture experiments with non-irradiated and irradiated CAFs were used to measure IL-10 production by mDCs. iDCs were also used as control for comparison. Results were analyzed with flow cytometer and results represents mean value obtained from four different CAF donors. Data was analyzed with Student`s T-test and p-value (*<0.05) was calculated between controls and non-irradiated CAFs. Results are presented as fold M0 controls.*

6 Discussion

It is generally believed that CAFs have an important role in the modulation of an anti-tumor immune response displayed in the tumor microenvironment [169]. Besides, DCs play a critical role in generating anti-tumor CD8 T cell immunity [170, 171]. Ionizing irradiation has been demonstrated to induce changes in the tumor stroma such as e.g., DNA damage, hypoxia, inflammation, cellular stress and cellular senescence [172]. Evidence from *in vitro* studies has indicated that CAFs is a radioresistant cell type and may survive high ablative doses of ionizing radiation [173]. However, radiation doses above 12 Gy induce permanent DNA damage responses and the concomitant development of irreversible cellular senescence [168]. Additionally, high radiation doses are able to influence the secretory profile of CAFs, thus affecting potential CAF-mediated paracrine signaling. However, little is known about how ionizing radiation could modulate CAF-mediated immunoregulatory effects on DCs. In this study, we showed that:

- CAFs down-regulate the expression of maturation markers on DCs trough paracrine signals and cell contact-dependent mechanisms
- DCs immune functions are curtailed when cultured with CAFs or CAF-CM
- CAFs down-regulate the expression of IL-12 in CAF-CM experiments, while the expression of IL-12 is upregulated in co-culture
- The secretion of IL-10 is enhanced by CAFs in co-culture
- Fractioned medium-high doses and to a lesser extent single high-dose irradiation can revert some of the CAF-mediated effects exerted on DCs

Tumor infiltrating immune cells are exposed to different factors in TME that may regulate their function [174]. CAFs are known for contributing to create this immunosuppressive environment, and inflammatory cytokines in CAFs secretory profile includes TGF-beta, IL-10, HGF and VEGF [91, 175].

We used different *in vitro* assays to characterize the influence of CAFs on DCs phenotype and function. Initially, we checked the influence of CAFs on DCs differentiation and maturation. One of the main differentiation markers for myeloid lineage cells is CD14, which is a protein crucial for immune recognition [176]. Monocytes are characterized by high expression of CD14, while iDCs and mDCs lose the expression of CD14 during differentiation and maturation stages. In our evaluation we also used CD1a and CD209, both surface receptors

expressed on iDCs related to antigen uptake [177-179]. Our controls showed that isolated monocytes displayed high expression of CD14 and low expression of CD1a and CD209 as expected. Further, iDCs had lower expression of CD14 and higher expression of both CD1a and CD209, which confirms the transdifferentiation of monocytes into iDCs. Our findings indicated that CAFs suppress to a large extent monocyte – iDCs conversion as showed by expression of differentiation markers.

Further, we analyzed if CAFs could block the maturation of DCs. iDCs are characterized by moderate expression of CD14 and high expression of CD1a and CD209, while mDCs have lost almost all expression of CD14 and shows moderate expression of CD1a and CD209. iDCs stimulated with the cytokine cocktail and incubate in the presence of CAF-CM shows enhanced expression of all surface markers with significant result for CD14. We also assessed CAF-mediated regulation of DCs co-stimulatory molecules CD80, CD86, CD40 and HLA-DR typically expressed on mDCs and used in communication with naïve $CD4^+$ T cells. We showed that CAFs restrained the maturation of iDCs significantly by down-regulation of CD80 and CD86. Our findings demonstrated that CAFs suppress the expression of co-stimulatory molecules CD80 and CD86 mainly through paracrine signaling. For the expression of CD40 and HLA-DR, we could observe that CAFs suppressed the expression of both surface receptors in CAF-CM and co-culture experiments.

The biology of DCs can possibly be altered by the secretome of CAFs. Studies have demonstrated that IL-10 can inhibit the maturation of iDCs, resulting in downregulated expression of CD80 and CD86 [180]. Corinti *et al* (2001) also provided evidence that culturing DCs with IL-10 blocked the maturation of iDCs [181] supported by studies were tumor-derived IL-10 is correlated with accumulating DCs with immature phenotype [182]. Wang et al (1995) showed that IL-10 has an inhibitory effect on the NF-κB pathway [183], and other studies have demonstrate that blocking of NF-κB signaling during DCs differentiation results in anergy and regulatory T cell activity [184]. There is evidence showing that TGF-β could suppress the maturation process [185, 186]. Mou *et al* demonstrated by using a mouse model that DCs maturation is restricted by TGF-β and also downregulate toll-like receptors [187]. VEGF may be involved in the suppression of maturation as well. Study shows that VEGF binds to Flt-1 receptor and inhibits the NF-κB pathway by decreasing the specific DNA binding of NF-κB [188]. This demonstrates that CAF-derived inflammatory molecules could have profound effects on DCs maturation.

For all assays regarding CAFs modulating effect on DCs functionality, we used both iDCs and mDCs in controls. iDCs are specialized cells for antigen uptake, while mDCs have a phenotype participates in migration to lymph nodes and for priming of naïve $CD4^+$ T cells. Our controls demonstrated that iDCs antigen capacity was much higher than mDCs while mDCs displayed better migratory and T cell priming ability compared to iDCs. The effect of CAFs modulation on DCs functions was in some cases quite high. For antigen uptake, we could observe that CAFs exerted their effect mostly through secreted molecules as was demonstrated in the experiments using CAF-CM were the antigen uptake was reduced compared to controls. In migration experiments, both CAF-CM and co-culture conditions blocked approximately 50 % of the migratory capacity of mDCs. There are some factors that are known to reduce the migratory capacity of DCs, such as IL-6, TGF-β, and VEGF, by changing the expression of chemokine receptors [189]. Other studies have investigated the correlation between TGF-β and the effect upon DCs migration, which demonstrated that TGF-β reduces the migratory capacity of mDCs *in vivo* [190, 191]. As for priming of naïve $CD4^+$ T cells, CAFs demonstrated to suppress T cell priming within 10 % in cells cultured with CAF-CM and 15 % in co-culture. Yang and Lattime (2003) demonstrated that IL-10 could suppress DCs priming of $CD4^+$ T cells in a mouse bladder carcinoma model [192]. These findings further support that CAF-secrete signal molecules contributes to suppress DCs function.

DCs are in addition to be important cells for the initiation of adaptive immune responses also crucial for maintaining immune homeostasis and can be considerate as gatekeepers of the immune system [193]. IL-10 is a pleiotropic cytokine with anti-inflammatory properties and can thereby suppress immune responses [194]. IL-10 is known to have immunosuppressive functions and can mediate inactivity of $CD4^+$ T cells in addition to reduce NK cells expression of inflammatory cytokines but stimulate proliferation of mature $CD8^+$ T cells *in vivo* [195]. The role of IL-10 in relation to cancer is controversial, since some studies suggest that IL-10 has a tumor-promoting role [196] while other studies indicates that IL-10 has an anti-tumor activity [197]. But it is generally believed that IL-10 is tumor-promoting, and an elevated level of IL-10 is usually correlated with poor prognosis for patients [198]. We wanted to investigate if CAFs could be responsible for the education of DCs towards IL-10 secreting DCs, which is an indication of a tolerogenic phenotype of DCs [199]. In fact, for experiments regarding paracrine signaling using CAFs supernatants, we did not observe an elevated production of IL-10 by DCs.

For DC/CAF co-culture experiments we could see that CAFs induced upregulation of IL-10 in DCs.

On the other hand, IL-12 is a pleiotropic, anti-inflammatory cytokine with pivotal immune-modulatory roles with important anti-tumor activity. IL-12 is known for inducing proliferation of NK cells and T cells, which results in the production of cytokines such as IFN-γ. IL-12 also activates STAT4, which improves the generation and activity of CTLs [200]. One of the main sources for IL-12 are antigen presenting cells, such as DCs [201]. We speculated that CAFs might downregulate the production of IL-12, due to the anti-tumoral nature of IL-12 [202]. We showed that CAFs blocks the secretion of IL-12 by DCs cultured with CAF-CM as we expected. But for the experiments in co-culture, we could see that CAFs actually increased the expression of IL-12. A possible explanation for the opposite result can be explained by CD40 – CD40L interactions in co-culture conditions, which activates TRAF6 and induces activation of p38 Kinase (MAPK9) and JNK (Jun Kinase) and upregulation of IL-12 by DCs. This was demonstrated in knockout mice that were unable to produce IL-12 in response to CD40L [138]. Another study demonstrated that both CD40L, mRNA and protein is produced by lung fibroblasts [203], which could explain why we could see increased expression of IL-12 by DCs in co-culture experiments due to the interaction of DCs surface markers CD40 and CD40L on fibroblasts.

Furthermore, we evaluated the effect of irradiation, applied as fractioned medium-high dose (3x6 Gy) or single high-dose (1x18 Gy) on CAF-mediated tolerogenicity on DCs. In this study, we observed some moderate changes in the expression of surface markers after CAFs had been irradiated with a few remarks. In the differentiation of monocytes into iDCs, we could observe a reverted expression of CD1a after CAFs had been irradiated with 3x6 Gy, although the result was not significant. And for CD14 in maturation from iDCs into mDCs, we could observe that both regiments of irradiation had a significant suppressive effect on the expression of CD14. As for co-stimulatory molecules CD80 and CD86, the expression was reverted after CAFs had been irradiated with 3x6 Gy with a significant result for both receptors in CAF-CM experiments. *In vivo* studies have demonstrated that irradiation of mice with medium-dose radiation enhance expression of CD80 and CD86 on DCs [204]. Same tendencies were observed with surface receptors CD40 and HLA-DR in CAF-CM and co-culture experiments

were irradiation with 3x6 Gy blocks or eliminate the immunosuppressive effects exerted by CAFs on DCs maturation.

In functional studies, we could see that irradiation of CAFs with 3x6 Gy improved the functionality of DCs in antigen uptake and migration with significant results. For priming assays, CAFs irradiation did not alter DCs priming of $CD4^+$ T cells. On the contrary, Lee et al. demonstrated using an *in vivo* melanoma model that ablative irradiation with single high-dose of 20 Gy improved T cell proliferation in draining lymph nodes [205]. This could demonstrate that irradiation *in vivo* may have different effect on the priming of $CD4^+$ T cells compared with *in vitro* model as we used in our experiments.

DCs can due to their highly functional plasticity exert both immunogenic and tolerogenic immune responses [206]. DCs with tolerogenic phenotype and functions are known as tolerogenic DCs (tolDCs) or semi-mature DCs [207]. DCs can be differentiated into tolDCs *in vitro* with growth factors such as IL-10, TGF-β and HGF. CAFs secrete these inflammatory cytokines, suggesting that CAFs can polarize DCs to become tolerogenic through pathways related to these signal molecules [105, 208, 209]. But the mechanisms for inducing DCs to become immunogenic or tolerogenic *in vivo* are not fully understood [210]. TolDCs are able to induce T cell tolerance through different approaches, including production of anti-inflammatory cytokines, induction of T cell anergy, suppress effector T cells, generation of Tregs through *de novo* differentiation or activating already existing Tregs [211]. TolDCs can be characterized by low expression of functional markers such as CD80, CD86, CD40 and MHC complexes [212, 213] and can resemble iDCs with poor immunogenic functions [214]. In this study, we could observe that there exists a communication between CAFs and DCs. From these findings we can further interpret that CAFs may educate DCs to become tolerogenic through CAFs secretome, but possibly also by cell-contact mechanisms on the foundation that CAFs are able to down-regulate the expression of functional markers for DCs and also the functionality of DCs. Elevated production of IL-10 by DCs could also indicate that CAFs induce tolDCs phenotype, but it has to be taken into consideration that the measured concentration of IL-10 from experiments was quite low and that *in vitro* experiments may be quite different from what could be the case *in vivo*.

Our group is continuing its research to determine regulatory factors secreted by CAFs that are involved in the modulation of different immune cells, including DCs. In this future research, exosome-related experiments is one of the focus areas. This includes isolation of exosomes from CAFs by ultracentrifugation followed by *in vitro* assays including surface markers and functional experiments for trying to determine if CAF-secreted exosomes or other soluble factors contributes to the immunoregulatory role that CAFs play. At this point, the results are still inconclusive, but we were able to observe tendencies from the results, suggesting that CAF-secreted exosomes may contribute to induce immunosuppressive effects.

Here in this study, we demonstrated that both fractioned medium-high dose and high-dose irradiation induce changes in CAFs resulting in altered immunosuppressive effect towards DCs regarding expression of functional markers, cytokine production and functionality. However, our data suggests that fractioned delivered irradiation promotes more favorable effects on CAFs immunomodulation regarding DCs compared to single-high dose irradiation. These results demonstrate that fractioned radiotherapy could be beneficial and clinically relevant for improving immune responses *in vivo* by modulating the immunosuppressive environment in tumor stroma.

7 Conclusions

In this study, we have demonstrated that there is a cross-communication between CAFs isolated from patients with NSCLC and DCs isolated from peripheral blood of healthy donors. A few main observations can be highlighted from this study: **(1)** Our results demonstrated that CAFs in experiments with CAF-CM and in co-culture promotes changes in DCs causing down-regulation of certain functional markers, **(2)** CAFs suppressed the function of DCs through secretion of molecules and cell-cell interactions, **(3)** Both fractioned medium-high dose (3x6 Gy) and single high-dose (1x18 Gy) irradiation of CAFs induces changes which in some cases lead to normalization of surface marker expression and improved the function of DCs.

The immune system is crucial for restraining cancer growth, but as it turns out, tumors are able to highjack the immune system and alter their functions. This can reduce normal immune cell functions, but also promote immune cells towards protumorigenic machinery that leads to tumor progression. Several approaches have been taken trying to figure out how to overcome the challenges correlated with cancer research due to the genetic instability of cancer. Cancer research has provided knowledge about mechanisms used by tumors for their approach to communicate with immune cells, which has attributed development of therapeutic drugs that target molecular mechanisms used by cancer to hinder their immunoregulatory role. CAFs are one of the major cell types found in tumor stroma that promote immunomodulation through secretion of various cytokines and chemokines. This has resulted in therapeutic strategies targeting CAFs and the pathways this cell type uses to utilize its regulatory functions. But to further improve and generate more effective therapeutic approaches related to CAFs, additional understanding about CAFs themselves and their secretory profile is needed. However, this work is quite challenging due to the vast heterogenic origin of CAFs. It is also believed that tumor stroma includes CAFs with different phenotypes which further increases the difficulties trying to understand and characterize this cell type.

Future studies to understand the correlation between CAFs and DCs could include: (i) target factors secreted by CAFs that induces a tolerogenic TME, (ii) determine possible DCs signaling pathways changed by CAFs involved in the tolerogenic phenotype (iii) using *in vivo* models (iv) gain more insight about other immune cells such as macrophages, NK cells and T cells, which could improve the overall picture of CAFs immunoregulatory role.

8 References

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