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Immunobiological properties of cancer stem cells isolated from colorectal cancer patients.

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1. Index

During the the first part of my PhD experience I have been working in the laboratory of Professor Marco Vanoni, at the University of Milano-Bicocca. This group has recently identified few RasGRF1-derived peptides displaying both *in vitro* and *in vivo* Ras inhibitory properties. Mutations of RAS genes are critical events in the pathogenesis of different human tumors and Ras proteins represent a major clinical target for the development of specific inhibitors to use in anticancer therapies. Several independent biological evidences, including Ras-GTP intracellular levels, ERK activity, morphology, proliferative potential and anchorage independent growth demonstrated that the over-expression of these peptides could revert the phenotype of K-RAS transformed mouse fibroblasts to wild type. Their uptake into mammalian cells throughout the fusion of the RasGRF1-derived peptides with the Tat protein transduction domain was assessed. Notably the Tat-fused peptides, chemically synthesized, retained Ras inhibitory activity: (i) they were able to interfere *in vitro* with the GEF catalyzed nucleotide dissociation and exchange on Ras and (ii) to reduce cell proliferation of K-RAS transformed mouse fibroblasts.

Subsequently I spent eight months in the laboratory of Professor Andrea Morrione, at Kimmel Cancer Center, Philadelphia, USA where I was involved in the study of the role of insulin-like growth factor receptor I (IGF-IR) in tumor transformation. We demonstrated that IGF-IR played an essential role in the promotion of cell growth and in the protection of cancer cells from apoptosis. The IGF-IR was found over-expressed in invasive bladder cancer tissues compared with non-malignant controls. Although activation of the IGF-IR did not appreciably affect their growth but promoted migration and stimulated *in vitro* wound closure and

invasion. These effects required the activation of the Akt and Mitogen-activated protein kinase (MAPK) pathways. For this reason we studied the inhibitory effect of RasGRF1-derived peptides (Tat-fused) on IGF-I-induced migration and invasion in human bladder cancer cells. Notably we observed that the two events were strongly reduced and this evidence supported the use of RasGRF1-derived peptides as model compounds for the development of Ras inhibitory anticancer agents.

All data concerning the work described above have been published in two manuscripts (Metalli *et al* 2010; Sacco *et al*, 2011) indicated in the Biography section. In this thesis I will describe the results obtained during the last one and a half year of my experimental work in the group of professor Giorgio Parmiani, at the Unit of Immuno-biotherapy of Melanoma and Solid Tumors, San Raffaele Scientific Institute, Milan, Italy.

2. Riassunto

Il carcinoma del colon-retto è la terza forma più comune di cancro e la seconda principale causa di morte nel mondo occidentale. Nonostante siano stati identificati nuovi agenti terapeutici per questo tipo di tumore al momento nessun trattamento si è rivelato risolutivo per pazienti con tumore in stadio avanzato.

Nell'ultimo decennio l'identificazione di cellule con proprietà di staminalità nei tessuti tumorali ha consentito di ottenere nuove informazioni riguardo all'origine e alla composizione dei tumori; infatti queste cellule sono ritenute responsabili dell'iniziale formazione del tumore, della sua progressione e della sua resistenza agli agenti terapeutici.

Recentemente nel nostro laboratorio cellule staminali tumorali (CSCs) sono state isolate da campioni di tessuto provenienti da pazienti con diagnosi di tumore al colon-retto. In parallelo è stata isolata e stabilizzata *in vitro* dagli stessi tessuti in presenza di siero fetale bovino (Fetal Bovine Serum, FBS) la controparte di cellule "non-CSC", definite come cellule FBS.

Lo scopo principale di questo studio è stato quello di effettuare una caratterizzazione immunologica di cellule staminali tumorali del carcinoma del colon-retto e di investigare se queste cellule possono rappresentare cellule bersaglio per protocolli di immunoterapia. Il fenotipo di queste linee cellulari è stato analizzato tramite analisi di immuno-fluorescenza e citofluorimetrica, mentre la loro effettiva tumorigenicità è stata verificata attraverso l'utilizzo di xenotrapianti in topi immunodeficenti (NOD/SCID).

Inizialmente sono stati analizzati i livelli di espressione di molecole associate al tumore del colon-retto e alle cellule staminali , tra le quali: Ep-CAM, HCAM, CEA, CD44, Aldefluor, CD133, SOX-2, Nanog e OCT-

4. Gli inoculi seriali in topi immunodeficenti hanno confemato il maggiore potenziale tumorigenico delle CSCs rispetto a quello delle linee FBS. Gli xenotrapianti generati dalle CSCs mostravano infatti ampie aree di necrosi rispetto ai tessuti tumorali generati dalle FBS, indice di un'intensa attività proliferativa e l'analisi di immunoistochimica relativa ad alcune delle principali molecole associate alle CSCs e al CRC, ha messo in evidenza come gli xenotrapianti generati dalle CSCs mostrassero un fenotipo più affine a quello del tumore primario di origine, rispetto alle linee FBS.

In seguito abbiamo focalizzato l'attenzione sull'espressione delle molecole MHC di classe I e di classe II, dei ligandi di NKG2D, di molecole immunoregolatorie e delle molecole coinvolte nel processamento e nella presentazione (APM) degli antigeni associati ai tumori.

Sia le CRC CSC che le linee FBS erano entrambe debolmente positive per l' MHC di classe I e negative per l' MHC di classe II, mentre I ligandi di NKG2D erano comunemente rilevati in entrambi i tipi cellulari. Le molecole appartenenti all'APM erano principalmente difettive nelle CRC CSC rispetto alle linee FBS. Successivamente abbiamo determinato se le linee cellulari isolate da CRC potessero esprimere alcune molecole, tra cui CTLA-4, PD-1, B7-H3, in grado di regolare negativamente la risposta dei linfociti T attraverso la modulazione del secondo segnale richiesto per la completa attivazione dei linfociti T. Tutte le CSCs e le linee FBS erano omogeneamente positive per CTLA-4 e PD-1 e, inoltre, esprimevano alti livelli di PD-L1 e B7-H3. In aggiunta abbiamo osservato che tutte le cellule analizzate esprimevano sia nel citoplasma che in membrana la citochina IL-4, mentre il recettore di quest'ultima (IL-4R)

era debolmente presente a livello della membrana delle CSCs e assente in tutte le linee FBS. Questi dati confermerebbero la probabile produzione autocrina da parte delle CSC e delle linee FBS di IL-4 come è stato recentemente proposto dal gruppo di Stassi (Todaro M *et al,* 2007). Inoltre, questa citochina potrebbe svolgere un ruolo immunomodulatorio nei confronti delle risposte mediate dai linfociti T e dirette contro il CRC . Inaspettatamente si è osservato che le linee FBS rilasciavano nel supernatante di coltura elevati livelli di prostaglandina-E2 (PGE2), al contrario queste molecole non venivano trovate oppure venivano rilasciate a bassi livelli nei supernatanti delle CSC.

Inoltre è stato verificata l'efficienza di induzione di risposte anti-CRC lmediate dai infociti T isolati dal sangue periferico di pazienti con tumore mediante la loro stimolazione *in vitro* con le CSCs o le linee FBS autologhe. In un paziente con CRC (#1076) è stato possibile isolare linfociti T di tipo TH1 (definiti sulla base della secrezione di IFN-γ) che mostravano riconoscimento debole delle CSC. Al contrario una più efficiente secrezione di IFN-γ veniva misurata in seguito alla stimolazione di queste cellule T con le cellule FBS. In un secondo paziente (#1247) è stato osservato un riconoscimento delle CSC in modo indipendente dalle molecole MHC, suggerendo una reattività di tipo NK. Questo risultato era anche confermato dall'analisi fenotipica dei linfociti che evidenziavano un 'arricchimento 20-30% di cellule di tipo NK (CD3- CD16+CD56+). Questi risultati correlano con l'osservazione che le CSC avevano una espressione difettiva delle molecole coinvolte nel processamento e nella presentazione degli antigeni associata alle molecole MHC.

Sulla base dei risultati ottenuti possiamo concludere che abbiamo isolato da tessuti di CRC cellule con proprietà di staminalità. Queste cellule

tuttavia confrontate con la controparte FBS, mostravano un minore potenziale di immunogenicità. Linfociti T anti-CRC sono stati isolati dai pazienti con tumore che tuttavia mostravano maggiore reattività verso le cellule FBS rispetto alle CSC. Nel caso di un paziente sono state isolate *in vitro* risposte immunologiche preferenzialmente di tipo NK. Le nostre osservazioni, quindi potranno essere utili per disegnare nuovi protocolli di immunoterapia per pazienti con CRC e sottolineano la necessità di identificare nuovi antigeni al fine di recuperare l'immunogenicità delle CSCs.

3. Summary

Colorectal cancer (CRC) is the third most common form of cancer and the second cause of cancer-related death in the Western world. Despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the treatments options available is curative in patients with advanced cancer.

In the last decade, several studies have shown that a small population of "stem cell like" cells, with the capacity for self-renewal, multipotency and high tumorigenic potential, could be isolated from tumors with different histological origin, including human CRC. These peculiar stem cells are believed to be responsible for tumor initiation, progression and resistance to therapeutic agents. Therefore, treatments that are design to eradicate tumor masses should be also targeted to CSCs.

Recently in our laboratory cancer stem cells (CSCs) lines and their fetal bovine serum (FBS)-cultured non-CSC pair lines were *in vitro* isolated from surgical specimens of patients with primary CRC cancer. In this study we performed an immunobiological characterization of CRC CSCs and investigated whether these cells can represent suitable targets for immunotherapy protocols. The phenotype and functional characterization of these cell lines was examined by IF and cytofluorimetric analysis.

Firstly the expression levels of CRC cancer and/or stem cell-associated molecules such as: Ep-CAM, CEA, HCAM, CD44, Aldefluor, CD133, SOX-2, Nanog and Oct-4 was assessed. Notably, the tumorigenic potency of CSCs was higher than that of FBS cells as shown by the serial dilutions and serial transplantations cell injection in immunodeficient mice. Moreover the xenografts generated by CRC CSC showed large areas of necrosis than tumor tissues generated by FBS cells and better matched the phenotype of the original tumor, as revealed

by an IHC analysis of CRC and CSC-associated molecules. In addition, the expression of MHC-I and MHC-II molecules, NKG2D ligands, immune regulatory molecules, antigen-processing machinery (APM) and tumor associated antigens (TAA) was examined. Both CRC CSC and FBS lines were found weakly positive for MHC-I and negative for MHC-II, while NKG2D ligands were commonly detected in both cells types.

The APM was mainly defective in CRC CSC versus FBS cell lines. We also investigated whether the expression of immune-regulatory molecules (CTLA-4, PD-1, B7-H3) can affect the immunogenicity of these cells. All CSCs and FBS cell lines were homogeneously positive for CTLA-4, PD-1 and, although at higher levels for PD-L1 and B7-H3. All these molecules acts as negative modulator of T-cells response because of the inhibition of the vital 'second signal' required for optimal T cell recognition and activation.

Interestingly, IL-4 was expressed by all the cell lines both in the cytoplasm and in the membrane while IL-4R was weakly detectable on the membrane of CSC and was absent in FBS lines counterparts. These data may confirm the possibility of an autocrine production by CRC CSC and also FBS line of IL-4, that may thus protect themselves by apoptosis as recently described by Stassi's group (Todaro M *et al,* 2007), this citockine may also induce an immunomodulatory role for T-cells mediated responses against CRC. Unexpectedly FBS tumor lines released in the supernatants high levels of prostaglandin-E2 (PGE2) that, on the contrary was weakly or absent in the supernatant of CSCs.

In addition, T lymphocytes isolated from the peripheral blood of CRC patients were stimulated *in vitro* with autologous CSCs or FBS, in order to assess of anti-CRC reactivity. In patient #1076 we could isolate mostly

TH1-mediated responses (detected by INF-γ release), showing weak reactivity against CSCs. On the contrary a more efficiently INF-γ release was detectable when T-lymphocytes were stimulated with FBS tumor cells. In a second patient (#1247), a MHC-independent recognition of CSCs was observed, suggesting an NK-mediated response. This result was also confirmed by the phenotype analysis of these lymphocytes that revealed the presence of 20-30% NK cells ($CD3^{-}CD16^{*}CD56^{+}$).These findings correlate with the defective expression of molecules involved in antigen presenting and processing machinery observed in CSCs.

Taken together our data we can conclude that we have isolate from CRC tissues cells with stemness properties, thus showing a lower immunogenicity compared with the FBS counterpart. T cell responses could be obtained in CRC patients directed against CSC and FBS cells, though with higher reactivity for FBS tumor cells, while in another patient NK-mediated responses could be isolated These findings may be useful for the identification of new agents that can efficiently rescue the immunogenicity of CSCs in order to be targeted by T cell-mediated immune response.

4. Introduction

4.1 Stem cell (SC) biology.

Stem cells are undifferentiated cells defined by an indefinite potential for self-renewal, multi-lineage differentiation, and long-life. Almost all adult tissues contain a small population (usually less than 1%) of tissue-specific stem cells that are responsible for tissue-homeostasis (renewal and damage repair) throughout the life-time of an organism (Clarke and Fuller, 2006).

4.1.1 Self-renewal.

Self-renewal is the ability of a cell to divide and produce an exact copy of itself (Ward and Dirks, 2007). Stem cells can divide asymmetrically or symmetrically (Figure 1). Through symmetrical division, the cell gives rise to two daughter cells that either retain the same stemness properties of the progenitor or start the differentiation program, losing the ability of self-renewal. The stem cell would be in this case, lost. When the stem cell undergoes asymmetrical division, two different cells arise: one similar to the progenitor, which stays in the same microenvironment (the stem cell niche) and another cell that will move from the surroundings and will turn into a precursor/progenitor cell. These cells undergo a specialization program, meaning that at the same time they differentiate and proliferate in an active way.

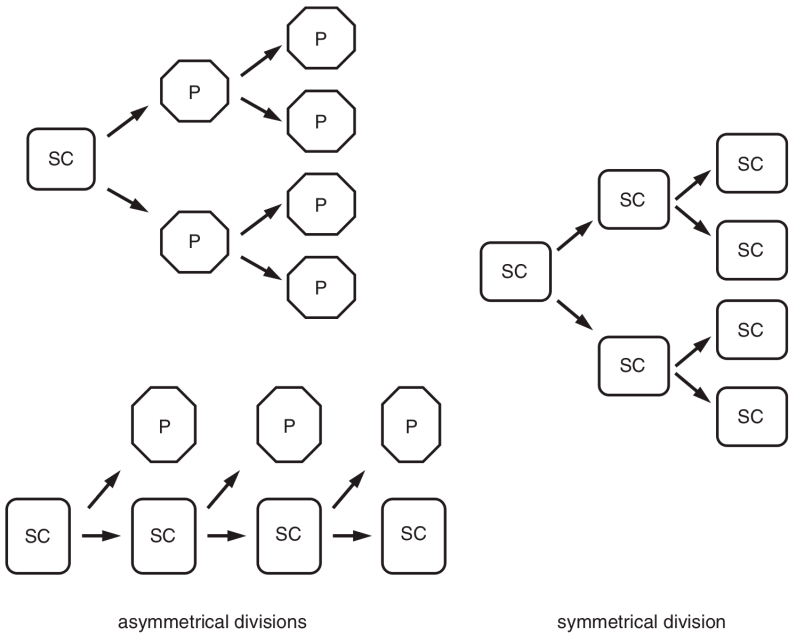

Figure 1: Stem cell division modalities. *SC* stem cell, *P* progenitor cell. (Papailiou *et al*., 2011)

4.1.2 Differentiation potential

There are various levels of potentiality that can be used to classify stem cells (Smith A., 2006):

- Pluripotentiality: a cell is capable of giving rise to any cell type of an organism, including some or even all extraembryonic lineages (i.e. embryonic stem cells).
- Multipotentiality: a cell is capable of given rise to all the different cell types of a tissue or tissues.
- Unipotentiality: a cell is capable of given rise to only a one cell type of a tissue (e.g., spermatogonial stem cells).

CSCs are, by definition, the cells capable of giving rise to all the differentiated cells that compose the tumors mass. However, in different types of tumors also Cancer Stem Cells with different potentials can be found. Some tumors produce a broad range of differentiated types among their descendants (e.g. teratomas) thus making it more likely that their CSCs share more characteristics with pluripotent stem cells. Other tumors, however, have a much more limited type of descendants (e.g. lymphomas), suggesting that their supportive CSCs could be closer to more limited multi- or uni-potential progenitors.

4.1.3 Quiescence/Long-Life

During quiescence some of the cellular activities stop, resulting in the activation of selective programs that make the cells refractory to differentiation. In a G0–G1 arrest the cells enter in a phase of low metabolism, in which the relation between the cells and the microenvironment is minimal. The reduced energy cost would allow the stem cells to have a long life. It has been proposed that the quiescent cells are not subject to the aging effect, a point that is also supported by the fact that the length of telomeres in stem cells remains constant after cell division (Huntly and Gilliland, 2005) The quiescence process in stem cells is reversible, like in cells which deprived of growth-promoting signals (i.e. serum withdrawal, contact inhibition). Stem cells are the only-long lived population of cells that is present in the tissues during all the time that is necessary for the accumulative mutations to cause the final oncogenic outcome. It is precisely their long life-span what makes them more susceptible to be the targeted by these fatal mutations. Quiescence of metastatic cancer stem cells is one of the postulated

mechanisms to explain the dormancy of metastasis that allows tumour relapse to appear years after clinical remission was first achieved (Aguirre-Ghiso, 2007).

Mutations within regulatory pathways could lead to the creation of stem cells with tumorigenic capabilities, the CSCs. It is important to understand in depth the characteristics of stem cells (shared by Cancer Stem Cells too), in order to search for a unique feature that would allow us to selectively target CSCs.

4.2 Colorectal cancer (CRC).

Colorectal cancer (CRC) is the third most common form of cancer and the second cause of cancer-related death in the Western world, leading to 655,000 deaths worldwide per year (Jemal *et al*., 2006). Despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the treatment options available is curative in patients with advanced cancer. A growing body of evidence is increasingly supporting the idea that human cancers can be considered as a stem cell disease. According to the cancer stem cell model, malignancies originate from a small fraction of cancer cells that show self-renewal and pluripotency and are capable of initiating and sustaining tumor growth (Boman and Wicha, 2008).

Experimental evidence has been generated that tumours may be organized as a hierarchy of phenotypically heterogeneous cell populations with divergent self renewal capacities, degrees of differentiation, and clonogenic potentials, and that cancer stem cells (CSC), which may comprise only a fraction of neoplastic cells but are nevertheless essential for its propagation, are found at the apex of this tumor cell hierarchy (Prince *et al*., 2007; Ricci-Vitiani *et al*., 2007) . CSCs are operationally defined as a clinical tumor specimen–derived cancer minority population, prospectively identifiable by a molecular marker or marker combination, that, unlike cancer bulk populations negative for the particular marker or set of markers, can initiate tumor formation and growth in immunodeficient hosts *in vivo*. In addition, CSC populations should exhibit the selective ability to (1) self-renew, demonstrated by serial xenotransplantation and/or genetic lineage tracking experiments *in vivo*, and (2) to differentiate and give rise to non-tumorigenic cancer bulk

components, thereby recapitulating the original patient tumor both morphologically and immunophenotypically in all serial xenografts (Reya *et al*., 2001). It is important torecognize that the CSC definition does not make any specific assumptions about the relative frequency of tumorigenic CSC populations or about any observed transdifferentiation plasticity of such cell subsets (Reya *et al*., 2001). The cancer-initiating cells or "cancer stem cells" were first identified in hematologic malignancies and most recently in several solid tumors, including CRC. The hypothesis of stem cell-driven tumorigenesis in colon cancer raises questions as to whether current treatments are able to efficiently target the tumorigenic cell population that is responsible for tumor growth and maintenance (Ricci-Vitiani *et al.*, 2009).

4.2.1 Colonic crypt organization

The colon is organized into four histologically distinct layers, including the mucosa, submucosa, muscle layer, and serosa. The inner most layer consist of mucosa tha includes the epithelium, lamina propria, and a thin layer of muscle. The epithelial layer, at the luminal surface, consists of a single sheet of columnar epithelial cells folded into finger-like invaginations that are supported by the lamina propria to form the functional unit of the intestine called crypts of Lieberkühn, wich contains approximately 2000-3000 cells (Booth and Potten, 2000).

The entire colon contains millions of self- renewing crypts, and it has been estimated that over 6 $x10^{14}$ epithelial cells are produced during the lifetime of an individual.

The terminally differentiated cells, which are found in the top third of the crypt, are continually extruded into the lumen. They are derived from

multipotent stem cells located at the bottom of the crypt. During asymmetric division, these cells undergo self-renewal and generate a population of transit-amplifying cells that, upon migration upward the crypt, proliferate and differentiate into one of the epithelial cell types of the intestinal wall. Three major terminally differentiated epithelial lineages are present within the crypt: the colonocytes, also termed absorptive enterocytes; the mucus-secreting goblet cells; and the less abundant peptide hormone-secreting enteroendocrine cells (Figure 2). Finally, Paneth cells, functionally similar to neutrophils, are scattered at the bottom of the crypt only in the small intestine epithelium and do not follow the downward migratory pathway.

Traditionally, colonic stem cells are considered to divide relatively slowly, and to leave the rapid proliferation necessary to populate the crypt to cells in the so-called transit- amplifying zone immediately above them.

The mechanisms implemented by the colonic niche are incompletely understood. The colonic niche cradles the bottom of a colonic crypt and is composed of pericryptal myofibroblasts (also known as intestinal subepithelial myofibroblasts), which surround the stem cell seat and are closely apposed to the surrounding basal lamina (Figure 2) (Powell D.,1999). Pericryptal myofibroblasts are thought to have various functions, including repair, organogenesis, mediation of epithelial–mesenchymal interactions, and control of extracellular matrix metab-olism . In addition, pericryptal myofibroblasts are thought to exert control over stem cells by influencing their self-renewal and differentiation via a variety of signaling pathways, among which the Wnt–β-catenin pathway is of central importance (Zeki *et al*., 2011).

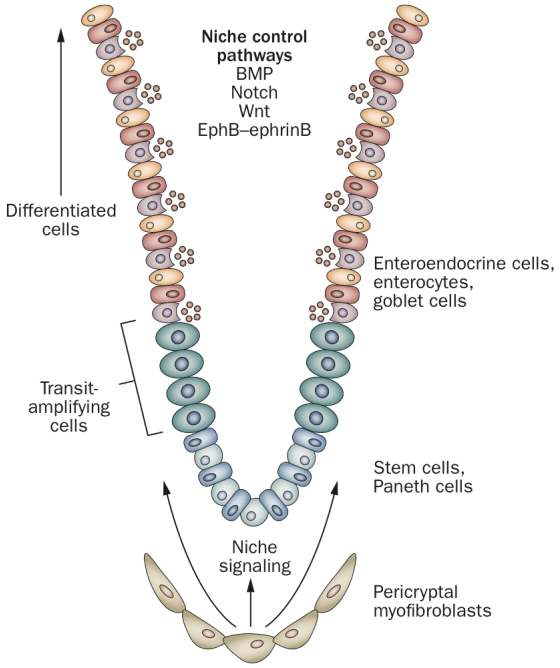

Figure 2: The colonic stem cell and its niche. (Zeki *et al*., 2011) .

The maintenance of the stem cell compartment as well as the transition from proliferation to differentiation are finely regulated by a relative small number of evolutionary highly conserved morphogenic pathways: WNT, Notch, bone morphogenetic proteins (BMPs) and Hedgehog.

4.2.1.1 WNT

Wnt signaling is involved in stem cell self-renewal, proliferation, differentiation and also in migration within the stem cell niche. In the canonical Wnt signaling pathway, extracellular Wnt proteins bind to the Frizzled (FZD) receptors in the membrane. After that, and together with

LRP5 and LRP6 co-receptors, the Gsk-3β and serine-threonine kinases are inhibited. Thus, β-catenin is not degraded and instead the unphosphorylated protein is first accumulated in the cytoplasm and then translocated to the nucleus. After that, the interaction of β-catenin with the transcription factors TCF/LEF leads to the activation of target genes such as Cyclin-D, DKK1, Myc, FGF20, WISP1 and CCND1, which are involved in regulation of cell proliferation and determination of cell-fate. Accumulation of β-catenin is associated with several solid tumors: melanoma, sarcoma, brain tumors and breast cancer, and mutated forms of the protein are found in several carcinomas (endometrial, prostate, hepatocellular) and colorectal cancer (Lobo and Zachary, 2000). In fact, it has been suggested that the constitutive activation of Wnt signaling could be the initiation episode or at least an important player in the development of many solid tumors (Clevers, 2006).

The therapeutic approaches targeting WNT pathway include small molecules mainly targeted against the Tcf-β-catenin complex (e.g., PKF118-310 or ZTM000990) and monoclonal antibodies (anti-WNT1 and anti-WNT-2), which have been proved to have anticancer effects in vitro (Katoh, 2007). Also, non-steroidal anti-inflammatory drugs (NSAIDS) like aspirin or indomethacin might contribute to the suppression of cancerous aberrant WNT signaling by a still unclear mechanism of action. Nowadays, many aspects of the WNT signaling pathway remain still unclear (the precise role of β-catenin in self-renewal, regulation of the dosage of WNT signaling), and further studies will be necessary to elucidate the specific targets for therapeutic treatment.

4.2.1.2 Notch

In normal conditions, NOTCH signaling is involved in the maintenance of self-renewal and in the determination of stem cell fate in several tissues. It acts at different stages of differentiation, from the regulation of embryonic development to adult maintenance of homeostasis (Chiba, 2006). There are four different NOTCH transmembrane receptors in humans, which can bind with five ligands. After ligand binding, the intracellular domain of NOTCH is cleaved and translocated to the nucleus, where it activates a cascade of transcriptional regulatory events that ultimately affects the expression of numerous genes, and leads to different phenotypic characteristics (Natarajan and FitzGerald, 2007). In most cases the pathologic activation of NOTCH signaling causes increased proliferation, limited differentiation, and prevents apoptosis in cancer cells.

Some characteristics of this pathway make it especially desirable as a target for anti-cancer therapy: (a) there is no enzymatic amplification step, meaning that the downstream effects of NOTCH activation are dose dependent. As result, therapeutic effects could be reached without completely turning off the pathway (Rizzo *et al*., 2008): (b) the intracellular half-life of NOTCH is very short, so inhibition treatment does not need to be constant, but could be discontinuous; (c) the effects of NOTCH are context-dependent, therefore the effects of inhibition could be different depending on each cell type. Researchers are looking for NOTCH signaling inhibitors as potential therapeutic agents that could in theory act at different levels. At the present time, inhibitors of the enzyme that cleaves NOTCH (γ-secretase; GIS) and some molecular antibodies against NOTCH receptor and ligands are in clinical trials.

4.2.1.3 Bone morphogenetic proteins (BMPs)

BMP belongs to a more complex family of ligands comprising BMP and transforming growth factor-β (TGF-β) family members, which share intracellular signalling through the SMAD proteins (Hardwick *et al.*, 2008) BMP2 and BMP4 are expressed by mesenchymal cells in the intestine and are suggested to halt proliferation at the crypt–villus border, allowing differentiation. To coordinate the segregation between proliferation and differentiation, BMPs are active at the top of the crypt, where differentiation occurs. BMPs are also produced at the crypt bottom, but here they are kept in check by BMP inhibitors (such as noggin) that are specifically expressed by the mesenchyme in the ISC region. Notably, BMPs also have dual functions and are involved in lineage fate decisions towards secretory cells (Auclair *et al.*, 2007). Through BMPs and noggin, the mesenchymal microenvironment thus secures a spatial organization in the crypt.

4.2.1.4 Hedgehog

The Hedgehog pathway is involved in developmental patterning control, playing an important role in proliferation, and regulation of self-renewal and survival of CSCs (Aoki *et al.*, 2003). There are three homologs of the Hedgehog (HH) gene: SHH, IHH and DHH. HH protein binds to its transmembrane ligand, Patched (PTCH), resulting in downregulation of Smoothened (SMO). In the nucleus, SMO activates the transcription factors GLI and HRK4.

Many evidences exist for the implication of the Hegdehog pathway (HH-GLI) in multiples forms of cancers affecting organs where this pathway plays a role in organogenesis, mainly brain, skin, esophagus, stomach,

pancreas, breast, lung, biliary tract, bladder, oral, and prostate (Varjosalo and Taipale, 2008). It has been described that in glioblastoma multiforme (GBM) self-renewal and survival of gliomasphere-forming cells relies on SMO and GLI activities (Bar *et al*., 2007) (Clement *et al.*, 2007).

One potential therapeutic treatment points to the use of antagonists of the molecules, which act downstream from the components of HH pathway.

Many aspects of HH signaling remain to be understood, and further studies will be necessary to throw some light into the mechanisms that regulate the developmental patterning control, and will help to design better targets against the HH pathway.

4.3 Stochastic and Cancer Stem Cell (CSC) model.

Two models have been suggested to explain the tumour progression, the stochastic model and the Cancer Stem Cell (CSC) model or hierarchical model (Lobo *et al*., 2007) (Figure 3). It is important to distinguish the cell of origin from the CSC. The cell of origin could be any cell type, e.g. a stem cell, a progenitor, or a differentiated cell (Figure 4). Once a cell acquires a mutation that affects its normal lifecycle and becomes the cell of origin, its cancerous development would follow according to the CSC or stochastic model. In other words even if the stem cell is the starting point of the tumour (cell of origin), it can form a tumour that adheres to the properties of the CSC or the stochastic model.

Until approximately 15 years ago, tumours were viewed according to the principles of the stochastic model. The most important characteristic of this model is that all cells of the tumour have equal ability to propagate the tumour by means of proliferation (Figure 3). The morphological heterogeneity observed when studying tumours is explained by genetic instability of the tumour cells, causing tumour cells to follow different aberrant differentiation pathways as well as cell intrinsic and extrinsic processes associated with tumour development, such as angiogenesis or Epithelial Mesenchymal Transitions (EMT). The main therapeutic consequence of this model is that in order to successfully treat a tumour, all of its cells need to be removed because all will be equally able to cause a relapse after therapy. In the last two decades the CSC model has put tumour heterogeneity in a new light. According to the CSC model, the tumour is viewed as an entity that can be studied applying the principles of stem cell biology. Thus, tumour cells maintain a hierarchical organization consisting of a population of self renewing cells at the

bottom of the hierarchy which will give rise to its progeny: 1) More CSC through self renewing cell divisions 2) Progenitor cells with limited proliferative potential 3) Differentiated cells with no proliferative potential (Figure 4). The clinical implication of this model is that by removing the CSC the tumour will no longer be capable of growing. So, instead of focussing on the reduction of the bulk of the tumour, specific therapies targeting the CSC will be required to eradicate the tumor (He *et al*., 2009).

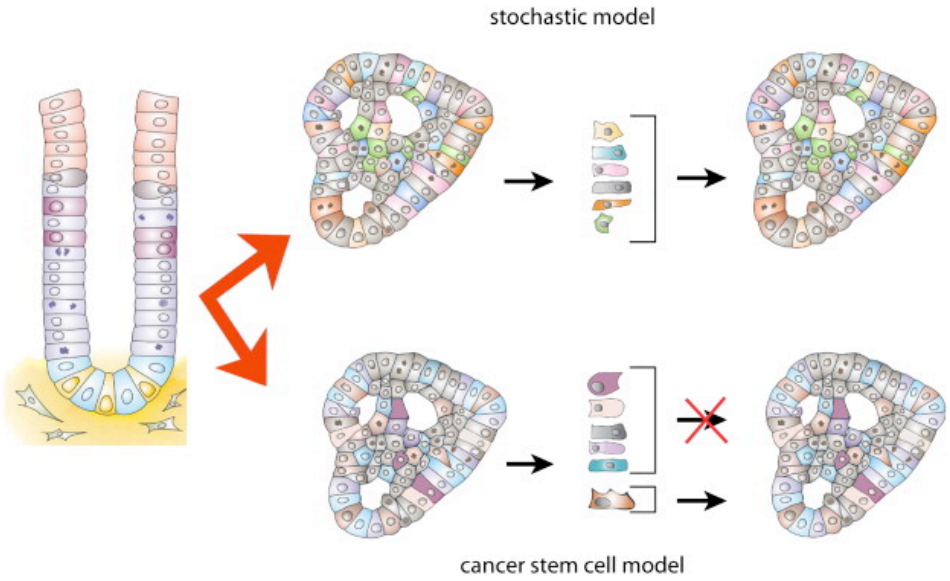

Figure 3: Comparison of the stochastic and the cancer stem cell model. In the stochastic model all cells have an equal probability to propagate the tumour. In the cancer stem cell model a unique population of CSC has the potential to propagate the tumour (Vries *et al*., 2010)

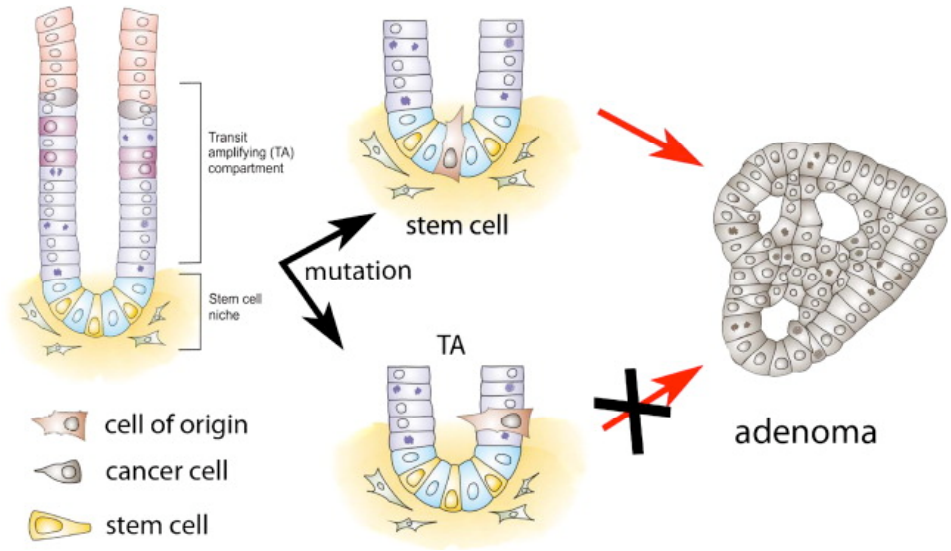

Figure 4: Tumor initiation by cells of origin. Recently, it was shown that adenomas induced by loss of APC originate from normal stem cells of the intestine, not the progenitor or differentiated cells (Vries *et al*., 2010).
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4.4 Do CSCs arise from SCs?

Tumors originate from the transformation of normal cells through the accumulation of genetic modifications, but it has not been established unequivocally that stem cells are the origin of all CSCs (Rapp *et al*., 2008). The CSC hypothesis therefore does not imply that cancer is always caused by stem cells or that the potential application of stem cells to treat conditions such as heart disease or diabetes, as discussed in other chapters of this report, will result in tumor formation. Rather, tumor-initiating cells possess stem-like characteristics to a degree sufficient to warrant the comparison with stem cells; the observed experimental and clinical behaviors of metastatic cancer cells are highly reminiscent of the classical properties of stem cells (Croker and Allan, 2008).

Given the similarities between tumor-initiating cells and stem cells, three main hypothesis are developed about the cellular precursor of cancer cells (Clark and Baudouin, 2006) (Figure 5).

4.4.1 First hypothesis: cancer cells arise from stem cells.

Stem cells are distinguished from other cells by two characteristics: (1) they can divide to produce copies of themselves, or self-renew, under appropriate conditions and (2) they are pluripotent, or able to differentiate into most, if not all, mature cell types. If CSCs arise from normal stem cells present in the adult tissue, de-differentiation would not be necessary for tumor formation. In this scenario, cancer cells could simply utilize the existing stem-cell regulatory pathways to promote their self-renewal. The ability to self-renew gives stem cells long lifespans relative to those of mature, differentiated cells (Croker and Allan, 2008). It has

therefore been hypothesized that the limited lifespan of a mature cell makes it less likely to live long enough to undergo the multiple mutations necessary for tumor formation and metastasis. Several characteristics of the leukemia-initiating cells support the stem-cell origin hypothesis. Recently, the CSCs associated with AML have been shown to comprise distinct, hierarchically-arranged classes (similar to those observed with hematopoietic stem) that dictate distinct fates (Hope *et al.*, 2004). To investigate whether these CSCs derive from hematopoietic stem cells, researchers have used a technique known as serial dilution to determine the CSCs' ability to self-renew. Serial dilution involves transplanting cells (usually hematopoietic stem cells, but in this case, CSCs) into a mouse during a bone-marrow transplant. Prior to the transplant, this "primary recipient" mouse's natural supply of hematopoietic stem cells is ablated. If the transplant is successful and if the cells undergo substantial self-renewal, the primary recipient can then become a successful donor for a subsequent, or serial, transplant. Following cell division within primary recipients, a subset of the AML-associated CSCs divided only rarely and underwent self-renewal instead of committing to a lineage. This heterogeneity in self-renewal potential supports the hypothesis that these CSCs derive from normal hematopoietic stem cells (Hope *et al.*, 2004). It should be noted, however, that the leukemia-inducing cells are the longest-studied of the known CSCs; the identification and characterization of other CSCs will allow researchers to understand more about the origin of these unique cells.

4.4.2 Second hypothesis: cancer cells arise from progenitor cells.

The differentiation pathway from a stem cell to a differentiated cell usually involves one or more intermediate cell types. These intermediate cells, which are more abundant in adult tissue than are stem cells, are called progenitor or precursor cells. They are partly differentiated cells present in fetal and adult tissues that usually divide to produce mature cells. However, they retain a partial capacity for self-renewal. This property, when considered with their abundance relative to stem cells in adult tissue, has led some researchers to postulate that progenitor cells could be a source of CSCs (Li *et al*., 2007) (Hope *et al*., 2004).

4.4.3 Third hypothesis: cancer cells arise from differentiated cells.

Some researchers have suggested that cancer cells could arise from mature, differentiated cells that somehow de-differentiate to become more stem cell-like. In this scenario, the requisite oncogenic (cancer causing) genetic mutations would need to drive the de-differentiation process as well as the subsequent self-renewal of the proliferating cells. This model leaves open the possibility that a relatively large population of cells in the tissue could have tumorigenic potential; a small subset of these would actually initiate the tumor. Specific mechanisms to select which cells would de-differentiate have not been proposed. However, if a tissue contains a sufficient population of differentiated cells, the laws of probability indicate that a small portion of them could, in principle, undergo the sequence of events necessary for de-differentiation. Moreover, this sequence may contain surprisingly few steps; researchers have recently demonstrated that human adult somatic cells can be

genetically "re-programmed" into pluripotent human stem cells by applying only four stem-cell factors (Takahashi *et al*., 2007; Yu *et al*., 2007).

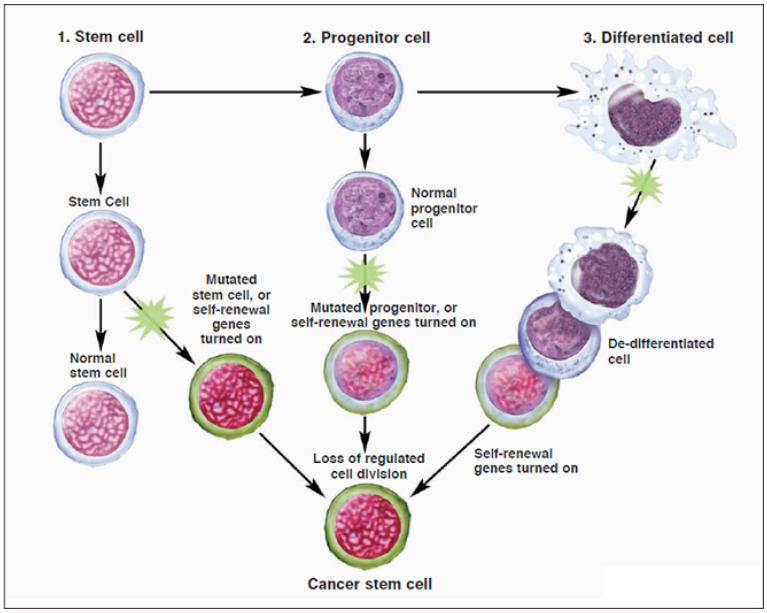


Figure 5: This figure illustrates 3 hypotheses of how a cancer stem cell may arise: (1) A stem cell undergoes a mutation, (2) A progenitor cell undergoes two or more mutations, or (3) A fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all 3 scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division (Boman and Wicha, 2008).

4.5 Identification of normal colon SCs markers.

During the past decade, considerable research effort has been made to identify the colon stem cell (Van der Flier and Clevers, 2009). Bromodeoxyuridine labeling was initially used to identify stem cells within the colon. This approach was based on the concept that stem cells divide infrequently and retain their DNA label for a longer period of time than the more rapidly dividing progenitor cells (Lipkin et al., 1963). Recently, however, this method has been replaced by the identification of ‘‘stemness’’ markers that allow stem cells to be isolated by flow cytometry (Table 1).

- The RNA-binding protein **Musashi-1** (Msi-1) was the first molecule identified as a putative human colon SC marker. Most information about its function came from studies in Drosophila, where it was found to be indispensable for asymmetric cell division of sensory organ precursor cells (Nakamura et al., 1994). Similarly, mouse Msi-1 was proposed to be required for asymmetric distribution of intrinsic determinants in the developing mammalian nervous system (Okabe et al., 1997). Msi-1 expression was then reported in mouse small intestine and in human colon crypt SCs (Nishimura *et al*., 2003; Potten *et al*., 2003). In mammals, Msi-1 is believed to maintain the undifferentiated state of SCs through the post-transcriptional control of down-stream genes.

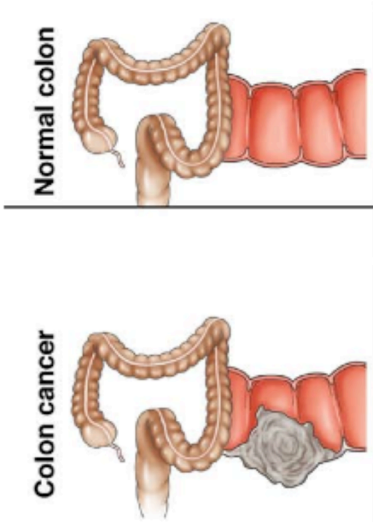
- Fujimoto et al reported that the **integrin subunit 1** (CD29) was a candidate surface marker for the proliferative zone of the

human colonic crypt, which includes SCs and progenitor cells (Fujimoto *et al*., 2002). They noticed that the cells located in the lower third of crypts expressed higher levels of CD29 than the cells in the remainder of the crypt. When crypt cells were isolated by flow cytometry based on CD29 levels, two cell populations that had different abilities to form colonies were identified.

- Barker's group used lineage-tracking experiments to identify a unique marker of normal colon SCs, the Wnt target gene **leucine-rich repeat-containing G protein-coupled receptor 5** (Lgr5) (Barker *et al*., 2007). Lgr5 is an orphan G-protein–coupled receptor of unknown function. In the intestine, it marks actively cycling cells, contradicting the concept that SCs are quiescent. However, Lgr5 did mark cells that were responsible for *in vivo* reconstitution of the complete small intestinal and colon epithelial lining. Moreover, a single Lgr5 cell from the intestine could regenerate a complete crypt-like structure *in vitro* (in Matrigel) (Sato *et al*., 2009).

- More recently, **doublecortin and CaM kinase-like-1** (DCAMKL-1), a microtubule-associated kinase expressed in postmitotic neurons, has been proposed as a putative colonic SC marker (May *et al*., 2008). DCAMKL-1 was found expressed in the same cells as Msi-1, but likely represented a subset of Msi-1–expressing cells. DCAMKL-1 cells were found apoptosis-resistant following radiation injury. Twenty-four hours after

ionizing radiation exposure, only few stem/progenitor cells were in fact removed by apoptosis, and the potential descendants were able to divide and, at least transiently, express DCAMKL-1 Importantly, DCAMKL-1 identified a population of quiescent cells, contrary to data obtained from Hans Clever's group, which identified a population of actively cycling SCs using the Lgr5 marker.



Marker	Other name	Function
Msi-1		RNA-binding protein
CD29	Integrin b1	Cell adhesion molecule
Lgr5	GPR49	Unknown, Wnt target gene
DCAMKL-1		Kinase
CD133	Prominin 1	Self-renewal, Tumor angiogenesis
ESA	EpCAM, BerEp4	Cell adhesion molecule
CD44	CDW44	Cell adhesion molecule, Hyaluronic acid receptor
CD166	ALCAM	Cell adhesion molecule
Msi-1		RNA-binding protein
CD29	Integrin b1	Cell adhesion molecule
CD24	HSA	Cell adhesion molecule
Lgr5	GPR49	Unknown, Wnt target gene
ALDH1	ALDC	Enzyme

Table 1: List of colon stem cell and cancer stem cell markers (Todaro *et al*., 2010).

4.5.1 CSCs associated markers.

Cancer cells with stem-like features were first observed in acute myeloid leukemia (AML) and later found in other tumor types. In Table 1 are listed the markers that have been proposed to characterize CRC SCs.

- One of the next CSC markers identified was **CD133**, a pentaspan transmembrane glycoprotein also known in humans as Prominin 1. The $CD133^{+}$ population is enriched in cancer-initiating cells in many tissues, including retinoblastoma, (Maw *et al.*, 2000) teratocarcinoma, brain tumor, kidney cancer, prostate tumor, hepatocellular, and colon carcinomas (Ricci-Vitiani *et al.*, 2007). Nonetheless, use of CD133 as a marker for identification and isolation of colon CSCs is a subject of debate; despite its use in isolating cell populations with cancer-initiating ability, studies have shown that CD133 is expressed by SCs and more differentiated progenitor cells (Shmelkov *et al*., 2008) CD133's function is unclear, although it is believed to have a role in asymmetric division and self-renewal. Within the intestine, CD133 would mark SCs susceptible to neoplastic transformation. These cells would be in fact prone to aberrantly activate Wnt signaling and such event would disrupt normal tissue maintenance leading to their aberrant expansion, resulting ultimately in neoplastic transformation of the intestinal mucosa (Zhu *et al*., 2009).

- Subsequently, Dalerba *et al* (2007) proposed **CD44** and the **epithelial cell adhesion molecule** (EpCAM) as CRC-SC-

specific markers, with further enrichment by **CD166**. Purified $CD44^{+}$/$EpCAM^{HIGH}$ cells injected into NOD/SCID mice resulted in high frequency generation of tumor xenograft. In contrast, $CD44^{-}$ /$EpCAM^{LOW}$ cells lack tumor-initiating activity (Dalerba *et al.*, 2007). Further sub-fractionation of the $CD44^{+}$/$EpCAM^{HIGH}$ cell population by using the mesenchymal stem cell marker CD166 increased the success of tumor xenograft.

- **CD24**, first described as a cell surface marker for several haematopoietic cell populations, is known to play a role in cell-cell interaction (Aigner *et al.*, 1998; Aigner *et al.*, 1997), adhesion and proliferation (Chappel *et al*., 1996). Many cancers were found to over-express CD24 (Jacob *et al*., 2004). Indicating that the protein can play an important role in tumorigenesis. However, its role in CSC biology remains undefined, especially because the expression level by which the CSC should be selected differs between tissue types. Together with CD24, CD29-positivity has been used to identify the colon CSC population (Vermeulen *et al*., 2008).

- The trascription factor **SOX2** belongs to group B of the Sox family and plays critical role in cell fate determination and is expressed in several malignant tissues (Gu *et al.*, 2007). It is involved in the later events of carcinogenesis such as invasion and metastasis, and plays a role in conferring a less differentiated phenotype in these tumors (Tsukamoto *et al*., 2005). Its known role in development, cell differentiation, and

proliferation suggest that this transcription factor may be relevant to the aberrant growth of tumor cells. Overexpression of SOX2 has been found in colon cancer compared to their normal tissue counterparts (Rodriguez-Pinilla *et al*., 2007).

- **OCT4** is a major POU-domain transcription factor that is mandatory for the self-renewal and pluripotency characteristics of embryonic stem (ES) cells and germ cells (Cheng *et al.*, 2007). Rare cells that express OCT4 were identified in several somatic cancers. OCT4 expression in the intestine causes dysplasia by inhibiting differentiation in a manner similar to that in the ES (Hochedlinger *et al*., 2005). Its overexpression has been found in primary colon cancer compared to their normal counterpart tissues (Yamanaka, 2008).

- Finally, in a more recent study, **aldehyde dehydrogenase 1** (ALDH1) has been proposed as a promising new marker for normal and malignat human colonic SCs (Huang *et al.*, 2009). ALDH1 is a detoxifying enzyme that oxidizes intracellular aldehydes and converts retinol to retinoic acid. Because of its function, it could protect SCs against oxidative insult, allowing for longevity and also modulate SCs proliferation. Huang et al (Huang *et al*., 2009) described subsets of $CD44^+$ or $CD133^+$ cells that were positive for ALDH1 and located at the base of the normal crypt. During colon tumor progression to carcinoma, the number of cells positive for all 3 markers (CD44,CD133, and ALDH) increased and were distributed further up the crypt axis.

Human cancer cells, isolated based on enzymatic activity of ALDH and injected into non-obese diabetic severe combined immunodeficient mice, formed tumors. Selection of $CD133^+$, $CD44^+$ cells with ALDH1 activity enriched somewhat the CSC population.

The identification of these and also new biomarkers for CRC-SCs it will be useful in order to improve the understanding of mechanism underlying tumor growth and progression.

4.6 T lymphocytes immune-regolatory molecules.

T lymphocytes of the adaptive immune system are able to recognize and specifically respond to an incredible variety of foreign and native antigens. To ensure an appropriate Tcell response, which is essential to eradicate pathogens and to maintain self-tolerance, T-cell activation is finely tuned by two independent signaling pathways. The first signal requires recognition of the antigen-bearing major histocompatibility complex (MHC) on the surface of antigen-presenting cells (APCs) by the corresponding antigen-specific T-cell receptor (TCR) on T-cells. The second signal, which is antigen independent, is delivered by costimulatory molecules of the B7/CD28 family. B7-1/B7-2:CD28/CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) signaling represents the best characterized co-stimulatory pathway (Abbas, 2003) (Figure 6). Engagement of B7-1 on APCs with CD28 on T-cells enhances T-cell proliferation and IL-2 production. In the absence of this simultaneous co-stimulatory signal, ligation of the TCR by an antigenic peptide results in T-cell dysfunction, intolerance or anergy. Apart from stimulatory signals that augment and sustain T-cell responses, co-stimulatory pathways also deliver inhibitory signals that down-regulate or terminate Tcell responses (Chen, 2004). Binding of CTLA-4 to B7-1 and/or B7-2 inhibits IL-2 synthesis and progression through the cell cycle leading to the termination of T-cell response. In the past two decades, new co-stimulatory ligands and receptors have been identified, including PD-1 (programmed death-1) and its ligands B7-H1 (programmed death-1ligand-1) and B7-DC (programmed death-1 ligand-2), ICOS (inducible co-stimulator) and its ligand B7-H2 (ICOS-ligand), and B7-H3, and B7-H4

(Greenwald *et al.*, 2005) (Figure 6). PD-1 is an inhibitory receptor expressed on activated T cells that regulates the peripheral T cells tolerance. In particular PD-1 is involved in the induction and maintenance of T cell's intrinsic unresponsiveness to previously encountered antigens. The two PD-1 ligands PD-L1 and PD-L2 differ in their expression pattern. PD-L1 is constitutively expressed on T and B cells, DCs, macrophages, mesenchymal stem cells, and bone-marrow derived mast cells. PD-L2 expression is much more restricted than PD-L1, and it is inducibly expressed on DCs, macrophages and bone-marrow derived mast cells (Keir *et al*., 2008).

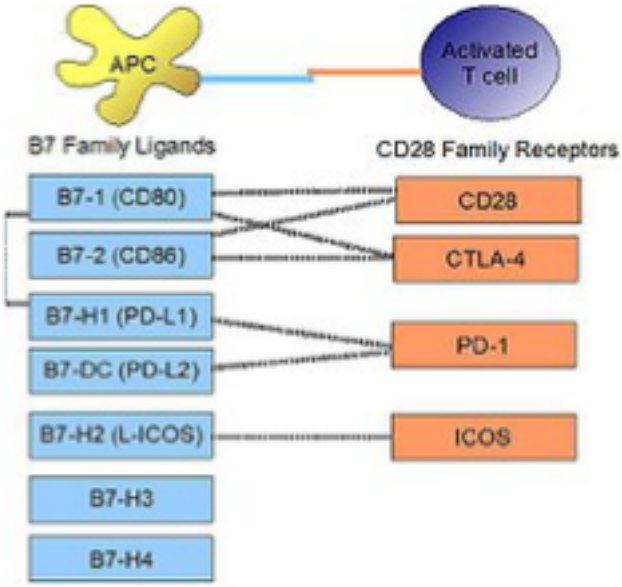


Figure 6: Schematic overview of co-stimulatory receptors and their ligands

Differently ICOS increases the proliferation of mouse and human T cells in response to TCR stimulation and also promotes cytokine production of

IL-4, IL-5, IFN-g, TNF-a, and GM-CSF by T cells. If CD28 appears to be key in the activation and differentiation of naive T cells early in the immune response, the inducible expression of ICOS, together with preferential induction of IL-10 by ICOS stimulation, contributes to amplify and/or regulate T cell responses. ICOSL is expressed on B cells, macrophages, CDs and on non-immune cells treated with TNF-alpha (McAdam *et al.*, 2000). At least B7-H3 identified in 2001, is not constitutively expressed on T-cells, natural killer (NK) cells, and APCs, but its expression can be induced on these cell types. In contrast to B7-1 and B7-2 whose expressions are mainly limited to immune cells such as APCs, B7-H3 protein is found widely expressed suggesting a more diverse immunological and probably non-immunological functions of B7-H3, especially in peripheral tissues.

Recently, B7-H3 expression has also been found in a variety of different human cancers, including colorectal cancer (CRC) indicating a possible involvement of B7-H3 in the regulation of antitumor immunity even if its exact role remains far from clear, because both stimulatory and inhibitory properties have been identified and both beneficial as well as adverse effects of B7-H3 expression in cancers have been reported (Loos *et al.*, 2010).

4.7 Immunotherapy.

In the last decades immunotherapy has emerged as one of the most promising treatment options for patients with metastatic disease, with sometimes striking but nevertheless often limited success. (Rosenberg *et al.*, 2008). Immunotherapy targets the induction or augmentation of anticancer immune responses. A major challenge has been to develop strategies that efficiently overcome tumor evasion of host immune responses and immunotherapeutic resistance (Mapara and Sykes, 2004). Strategies for enhancing antitumor immunity can be broadly categorized into: (1) non-specific immunomodulation approaches aimed at activating the host's immune response, (2) active immunization procedures directed at sensitizing the immune system against the autologous cancer (e.g.,cancer vaccines with whole cells, peptides, or immunizing vectors), and (3) adoptive cell transfer of *ex vivo* expanded autologous or allogeneic lymphocytes with tumor target specificity (Rosenberg *et al.*, 2008).

4.7.1 Non specific immunomodulation approaches.

Non specific activators of tumor-reactive lymphocytes approved by the US Food and Drug Administration include the up-regulation of immune reactivity by general immune stimulation (such as the administration of interleukin (IL)-2 or interferon (INF)-α (Rosenberg *et al.*, 2008; Schuchter, 2004) (Schuchter, 2004). However, most of the responses to mono-therapies with INF-α and IL-2 have been partial, with complete response rates ranging from approximately 5–15% (Schuchter, 2004). In the clinic, an obstacle of IL-2 immunotherapeutic regimens has been the

simultaneous increase in T regulatory (Treg) cells thought to contribute to poor treatment response (Powell et al., 2007). Depletion of the Treg cell pool may therefore represent a very promising approach to enhance immunotherapeutic efficacy, as indicated by findings in preclinical models(Jones *et al*., 2002). More recently, additional immunotherapeutic agents have been developed that target critical regulatory elements of patient immune cells to enhance their antitumor cytotoxic efficacy (Kirkwood *et al*., 2008).

These include monoclonal antibodies that functionally block negative regulators of lymphocyte activation, including CTLA-4, PD-1, and TGF-β (Fong and Small, 2008). Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is part of a large family of molecules that are involved in the activation or inhibition of T cell immune responses. CTLA4 is expressed on both CD8 and CD4 T cells, including the FOXP3+ regulatory T cells (Bao *et al*., 2008). The role of CTLA4 as an attenuator of an immune response was demonstrated in mice that were deficient in CTLA4. Two fully human antibodies have been developed and tested in metastatic melanoma. Tremelimumab and ipilimumab (Yervoy; Bristol-Myers Squibb) both showed objective response rates of approximately 10% (Frank *et al*., 2005) in early clinical trials. Interestingly, these responses were durable, sometimes lasting for years. Infusion of these antibodies was associated with the development of immune-related adverse reactions, such as colitis, dermatitis, hepatitis and endocrinopathies (such as thyreoiditis and hypophysitis), thus illustrating the role of CTLA4 in maintaining peripheral tolerance.

4.7.2 Active immunization procedures.

Active immunization with cancer vaccines (using whole cells, peptides, antigen-pulsed APC or known targets of immune reactivity) is an attractive therapy approach because of its ease of administration and lack of toxicity; however, no approach has yet been developed that can reproducibly mediate the regression of metastatic cancer at clinically meaningful levels (Pilla *et al.,* 2009) with the possible exception of anti-prostate cancer and anti-melanoma vaccines recently presented in the American Society of Clinical Oncology (May 2011). In light of the CSC concept, it would appear, however, more promising to target markers specific to the CSCs compartment (Parmiani *et al.,* 2007).

4.7.3 Adoptive cell therapy (ACT).

Adoptive T cell therapies are based on the infusion of large numbers of tumour-specific T cells. These tumour-specific T cells can be derived from the tumour environment (such as tumour-infiltrating lymphocytes (TILs), from peripheral blood or they can be genetically modified to express a high affinity anti-tumour T cell receptor (TCR).

Current clinical protocols for ACT requires the generation of highly avid tumour-antigen-reactive T cells (Figure 7). Tumour-specific T cells, can be efficiently isolated *ex vivo* from lesions using high levels of interleukin-2 (IL-2) and then TILs are successively selected for their ability to secrete high levels of interferon-γ (IFNγ) when cultured with autologous or allogeneic MHC-matched tumour-cell lines. (Rosenberg *et al*., 2008)

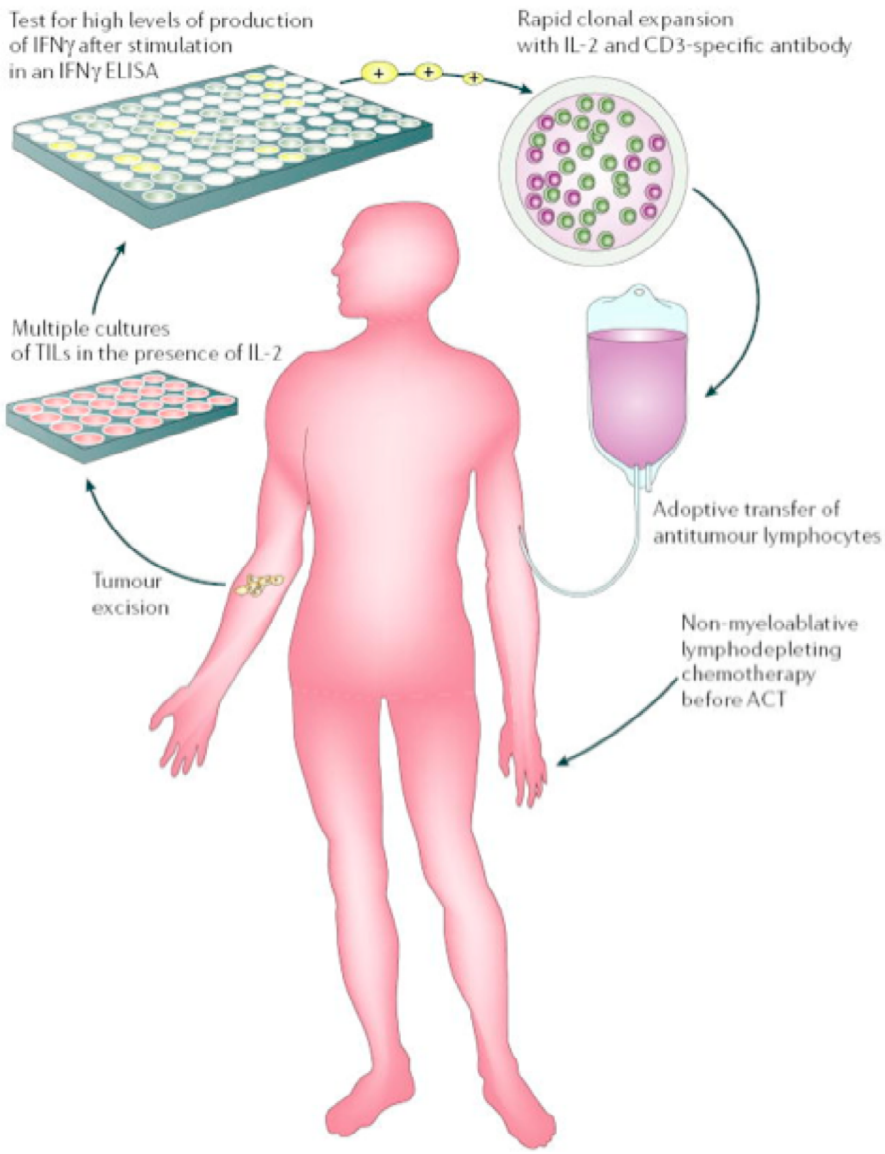


Figure 7: Current clinical protocols for adoptive cell therapy (Gattinoni *et al*., 2006).

4.8 An overview on colorectal carcinoma treatments.

Like most epithelial solid tumours, CRC has long been considered poorly immunogenic and substantially refractory to immunotherapy. This opinion was based on indirect data from: (a) epidemiological studies on lack of spontaneous regression of cancer; (b) *in vitro* studies on tumour infiltrating lymphocytes (TIL); (c) a first generation of clinical trials of immunotherapy in colorectal cancer patients. In fact, while in known immunogenic tumours, such as melanoma, primary lesions can sporadically undergo spontaneous regression associated with tumour infiltration by immune effectors, in CRC spontaneous regression is only exceptionally observed, and does not appear to be associated with an immune response (Dalerba *et al.*, 2003). Moreover, classical immunotherapeutic interventions known to be active against melanoma, such as systemic administration of cytokines (IL-2, INF-alpha) or adoptive transfer of autologous lymphocyte effectors (LAK, lymphokine-activated killer cells; TIL), have proved ineffective in CRC (Wolmark *et al.*, 1998). During the last decade, however, continuous progress in the molecular characterisation of T cell-defined tumour associated antigens (TAA) and in methods allowing detection of antigen-specific T cell responses have slowly modified the scientific community's perspective on this issue. Indeed, several different lines of evidence indicate today that also epithelial solid tumours may express TAA recognised by T cells, and that anti-tumour immune responses indeed may take place in colorectal cancer patients, influencing patient prognosis and shaping the tumour immunological profile.

4.8.1 Prognostic role of intra-epithelial $CD8^+$ T cells in colorectal carcinoma.

Cytotoxic T lymphocytes (CTLs), classically $CD8^+$ T-cells, represent the type of immune effectors that can more reliably be considered as a sign of a systemic anti-tumour immune response. In colorectal carcinoma several studies have focused on lymphocytes and have indicated that the presence of lymphoid infiltrates might have a positive prognostic role (Leo *et al.*, 2000). A key to understand the complex and contradictory series of data collected in colorectal cancer could reside in accurate and qualitative classification of inflammatory infiltrates, taking into careful consideration both the type of recruited immune effectors (T cells vs. other bystander effectors) and their anatomical localisation within the tumour tissues (intra-epithelial vs. stromal or peri-tumoural). Recently, Galon and his group, supporting the hypothesis that cancer development is strongly influenced by the host's immune system, observed that a strong infiltration of memory T cells with a Th1 and cytotoxic pattern is the strongest predictor for recurrence and metastasis in colorectal cancer (Fridman *et al.*, 2011). Indeed the presence of cytotoxic CD8+ and memory $CD45RO^+$T at level of both tumor center and both invasive margins will be inversely proportional to a disease relapse (Bindea *et al.*, 2011).

4.8.2 Adoptive immunotherapy effects in colorectal carcinoma.

In colorectal carcinoma, adoptive immunotherapy has been attempted with INF-γ activated macrophages and with IL2-activated lymphocyte effectors, such as LAK or TIL, without showing substantial clinical efficacy. Low frequency of anti-tumour T cells in lymphocyte preparations (Parmiani, 1990) and escape of the T cell response by tumour cells due to HLA-loss variants (Marincola *et al*., 2000) are two possible explanations of the low rate of success observed in T cell adoptive immunotherapy. A strategy to overcome both low yields in harvesting TAA-specific T cells and loss of class I HLA by tumour cells could be to collect large numbers of unselected T cells and to provide them with artificial receptors allowing recognition of tumour cells in a non-HLA and non-TCR restricted manner. Among the best characterised examples of such artificial receptors are the so-called T-bodies, chimeric receptors composed of antibody-derived variable regions as extracellular recognition domains, joined to the intracellular domains of the signalling subunits of different lymphocyte antigen receptors. Regarding colorectal carcinoma, different forms of T-bodies have now proven effective in redirecting the cytotoxic activity of T cells against CEA expressing tumour cells *in vitro* (Nolan *et al*., 1999).

4.9 Tumor immune escape mechanisms.

It is now well established that the body's immune system is capable of detecting, responding to and even eliminating tumours. For clinically apparent tumours it is thought that a 'lapse' of immune surveillance occurs, which consequently leads to tolerance of the growing nascent cancer. Since tumour cells express 'corrupted' versions of self molecules, a degree of tolerance is to be expected; however, various mechanisms exist to allow malignant cells to avoid immune detection and deletion (Dunn *et al.*, 2004). These can be generated by the transformed cells themselves or, more disconcertingly, by the immune system. A basic overview of the methods are now discussed:

- The nature of antigens, how they are processed by intracellular degradation pathways and presented as peptides bound to MHC molecules will determine the ability of the immune system to recognise their expression on tumours. Tumour cells that posses lower levels of expression of MHC avoid detection by T cells. This can be brought about by direct mutation of MHC-encoding genes or through changes to other molecules associated with antigen presentation, such as transporter-associated protein (TAP) or β-2-microglobulin (β2M) (Chang *et al.*, 2006; Setiadi *et al.*, 2007) (Figure 8). Deficiency in HLA class-I expression has a significant impact, since this molecule presents self-antigens, so obviously tumours will display properties to reduce this action (Cabrera *et al.*, 2003). This may be further compounded by problems such as low T cell avidity and affinity of the MHC-peptide complex for the

T cell receptor (TCR), which affect T cell recognition of peptides and the level of the immune response generated against them (McKee *et al.,* 2005).

- The loss of co-stimulation by B7.1 and B7.2 or tumour regulation of CTLA-4 or can prevent the generation of the vital 'second signal' required for optimal T cell recognition to occur (Macian *et al.*, 2004). In addition studies relating PD-L1 (B7-H1) expression in tumors show that this molecule may facilitate advancement of tumor stage and invasion into deeper tissue structures and its expression strongly correlates with bad prognosis (Keir *et al*., 2008).

- B7-H3, one of the recently identified members of the B7/CD28 superfamily serving as an accessory negative modulator of T-cell responses. Recently, B7-H3 expression has been reported in several human cancers, including CRC, indicating an additional function of B7-H3 as a potential regulator of antitumor immunity and also proposed as a target for anticancer candidates (Loos *et al*., 2010).

- Extensive research has drawn attention to the action of immuno-suppressive cytokines in contributing towards immune escape. Malignant cells have been shown to secrete high levels of specific cytokines, for example transforming growth factor β (TGF-β), IL-4, IL-10 and IL-17 amongst others (Figure 8). Paradoxically, in the initial stages of tumorigenesis, these cytokines may promote anti-

tumour responses; however, after prolonged periods, they can aid cancer progression (Li *et al.*, 2009; Nagaraj and Datta, 2010).

- Cancer cells may increase their resistance to cytotoxic T cell (CTL) attack by increasing the expression of cell surface molecules such as Fas-ligand (Fas-L/APO-1-L/CD178) (Figure 8). When ligated to its respective surface receptor, Fas (CD95), Fas-L initiates pro-apoptotic signalling within the Fas-expressing cells, leading to apoptotic cell death (Minas *et al.*, 2007). T cells express Fas as a safeguard to ensure that they can be destroyed in order to avoid potential autoimmune reactions; tumours therefore have the capacity to exploit this mechanism to ensure their own survival. However, while this phenomenon has been observed *in vivo*, there appears to be controversy with regard to this concept (Maher *et al.*, 2002).

- One of the more sinister aspects of host immunity–tumour relationship results from evidence that the immune system can contribute to tumour initiation and progression. This is evident from the role of regulatory T cells (TReg), a specific subset of T lymphocyte that act as inhibitors of adaptive immunity (Groux, 2001; Sakaguchi *et al.*, 2010), which exist naturally in the T cell pool or can be induced following immune stimulation (Figure 8). T reg cells inhibit the action of other T cell populations using direct contact, utilising the Fas-L/Fas interaction or perforin/granzyme-induced apoptosis or via the production of different immuno-suppressive cytokines, such as IL-10 (Strauss

et al., 2009). There is a strong belief that during immune activation, the homeostatic action of T reg cells acts to allow tumours to arise, unchecked by CD8+ or CD4+ antitumour responses and that tumours may promote cytokines such as TGF-β and IL-17 to induce T reg cells, thus subverting tumour immuno-surveillance (Chen *et al.*, 2003).

- NKG2D is a potent activating receptor expressed by NK cells, α/δ^{+}T and γ/δ^{+}T lymphocytes and macrophages and its ligands are the stess-inducible MHC class-I related molecules MICA, MICB and ULBP-1-4.NKG2D ligands are stress-response genes and are upregulated by stimuli such as DNA damage, heat-shock, and shifts in hormone levels. Soluble form of NKGD2L can be release by tumor cells by common cleavage process mediated by metalloproteases, and this represents one of the immune mechanisms leading T and/or NK cells to recognize tumor cells (Maccalli *et al.,* 2007). Althogh multiple strategies are used by cancer cells in order to prevent NKG2D mediated killing. The first and most obvious strategy is down-modulation of NKG2D ligands.

- Prostaglandin E2 (PGE2) is a bioactive lipid that mediates a wide range of physiological effects and plays a central role in inflammation and cancer. PGE2 is generated from arachidonic acid by the sequential actions of the cyclooxygenases (COXs)-2. An increased release of prostaglandin E2 (PGE2), as a result of overexpression of cyclooxygenase (COX)-2, has been observed

in many tumors, included CRC. Elevated levels of PGE2 secretion as well TGF-β and Il-10 inhibits dendritic cell maturation, resulting in the abortive activation of naive CD8+ T cells (Ahmadi *et al.,* 2008). In addition It has been demonstrated that COX-2 specific inhibitors reduces the proliferation and the invasiveness of human glioma cells *in vitro* (Joki *et al.,* 2000).

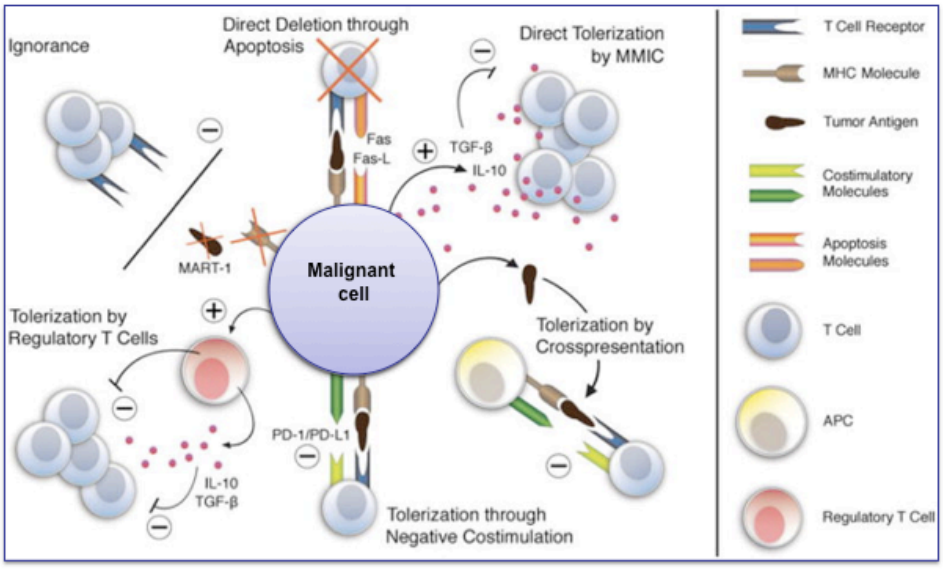


Figure 8: Basic overview of specific mechanisms of immune escape utilised by tumors (Schatton and Frank, 2009).

4.10 CSCs immune-escape mechanisms.

Taken together with the findings of higher rates of cancer development in immuno-compromised patients and animal models (Grulich *et al*., 2007), these results lend support to the notion that an intact host immune system may be able to eliminate transformed cells to prevent tumorigenesis at early stages of disease (Mapara and Sykes, 2004). This "concept of immuno-surveillance" may thus provide a potential explanation for the relatively low frequency of tumor development in healthy individuals. At the same time, the only a restricted minority of tumor cells, that is, the CSC, may possess the phenotypic and functional properties to evade host immuno-surveillance and immuno-mediated rejection in immunologically intact individuals. An immuno-selection of CSC populations that would be expected to be more capable of surviving in an immuno-competent host compared to tumor bulk components might be especially relevant in a highly immunogenic cancer (Rosenberg *et al.,* 2008).

Recentely Di Tomaso *et al.* (2010) performed an immunobiological characterization of glioblastoma multiforme (GBM) CSCs isolated from human tumor specimens. They observed that both GBM CSC and FBS lines were weakly positive and negative for MHC-I, MHC-II, and NKG2D ligand molecules, respectively. Antigen-processing machinery molecules were also defective in both cell types. Up-regulation of most molecules was induced by IFNs or 5-Aza deoxycytidine, although more efficiently in FBS than in CSCs. Patient T-cell responses, mediated by both TH1 and the TH2 subsets, against autologous CSC could be induced *in vitro*. In addition, CSC but not their paired FBS tumor lines inhibited T-cell

proliferation of healthy donors. Notably, a differential gene signature that was confirmed at the protein levels for some immunologic-related molecules was also found between CSC and FBS lines. These results indicate lower immunogenicity and higher suppressive activity of GBM CSC compared with FBS lines.

Furthermore Stassi et al. (Todaro *et al.*, 2007) isolated and characterized such cells from colon carcinomas using the stem cell marker CD133 that accounts around 2% of the cells in human colon cancer. The CD133+ cells grow *in vitro* as undifferentiated tumor spheroids, and they are both necessary and sufficient to initiate tumor growth in immunodeficient mice. Xenografts resemble the original human tumor maintaining the rare subpopulation of tumorigenic CD133+ cells. Further analysis revealed that the CD133+ cells produce and utilize IL-4 to protect themselves from apoptosis, through the upregulation of antiapoptotic genes, such as: cFLIP, PED and Bcl-x_L.

Consistently, treatment with IL-4Rα antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective sensitization of CD133+ cells. In conclusion these studies suggest that colon tumor growth is dictated by stem-like cells that are treatment resistant due to the autocrine production of IL4.

5. Materials and methods

5.1 Tissues and cell cultures.

Tumor lesions were obtained from patients with a diagnosis of primary colorectal cancer admitted for surgery at the San Raffaele Hospital, Milan, Italy.

CRC CSCs were isolated *in vitro* by mechanical dissociation of tumor specimens and were propagated in the form of spheres. Briefly, tumor cell suspensions were cultured in tissue culture 6-well plate at clonal density (2,500-5,000 cells/cm^2) in DMEM/F12 medium containing (growth factor dependent, GFD) or not (growth factor independent, GFI) 50 ng/ml of epidermal growth factor (EGF; Peprotech) and 20 ng/ml of fibroblast growth factor-2 (b-FGF-2; Peprotech) as previously described by G Galli et al, 2004. We used the sphere approach represented by a serum-free selective culture system in which most differentiated cells rapidly die, whereas stem cells respond to mitogens, divide and form spheres that can be dissociated and re-plated to generate secondary spheres. This process can be repeated by serial sub-culturing, which results in an exponential increase in the total number of cells and spheres that are generated.

Cultures were collected every 3 days and cells were dissociated into a single cell suspension followed by re-plating with fresh medium. When enough tumor tissue was available (patients #1076, 1247, 11011, 10595, and 14583), part of the tissue dissociation was plated in the presence of RPMI 1640 supplemented with 10% (FBS; Biowittaker, Lonza), hereafter denominated FBS tumor cells. These established cell lines were used in parallel with CSCs for all the experiments, representing their non-CSC

pair lines. FBS tumor cells displayed adherence dependent growth and showed differentiated morphology.

5.2 Multipotency properties of CRC CSCs.

CRC CSCs were cultured with medium containing 10% FBS, representing the differentiating agent, for up to 10 days. If cells show multipotency properties, initially they loose the ability to grow in suspension in the form of spheres and then they display adherence dependent growth; thus showing a differentiated morphology similar to the FBS tumor cells.

5.3 Immunofluorescence and flow cytometry analysis.

CSC phenotype characterization has been performed with the following antibodies: anti-EpCAM sc-51681 (Santa Cruz Biotechnology, Inc), anti-HCAM sc-51611 (Santa Cruz Biotechnology, Inc); anti-CD24 SN3 (Novus Biological); anti-CD133 293C3 (Milteny Biotech); anti-CEA (Novus Biological); anti-SOX2 245610 (R&D Systems, Inc.); anti-Oct-4 252-372 (BD Pharmingenand); anti-Nanog M55-312 (Becton Dickinson) and Aldefluor (Stem cells Technologies Kit).

The expression of HLA molecules was detemined using: HLA class I (Becton Dickinson) and HLA class II (Becton Dickinson).

The expression of Antigen Presenting Machinery (APM) molecules or of NKG2DLs, MICA, MICB, and ULBPs, by CRC CSC cell lines was determined by using the purified mAbs provided by Prof. Soldano Ferrone, Hillman Cancer Center, University of Pittsburgh Cancer

Institute, Pittsburgh, USA or by Amgen, respectively (Bandoh N. *et al*., 2005; Maccalli C. *et al*, 2007).

The expression of the immune-regulatory molecules has been performed with the following antibodies: anti–CTLA-4 BN13 (Becton Dickinson); anti–PD-1 MIH4 (Becton Dickinson); anti–PD-L1 MIH1 (Becton Dickinson); anti–PD-L2 MIH18 (Becton Dickinson); anti–B7-1 L307.4 (Becton Dickinson); anti–B7-2 2331 (FUN-1) mAb (Becton Dickinson); B7-H2 552502 (BD Pharmigen); B7-H3 185504 (R&D System Inc); IL-4 8D4-8 (BD Pharmigen) and IL-4R L8017 (BD Pharmigen).

The expression of these molecules by CSC and FBS tumor cell lines was determined by IF and flow cytometry (FACS CANTO HTS, Becton Dickinson). The phenotype characterization of T-cell lines was carried out by multicolor immunofluorescence and cytofluorimetric analysis with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD56, anti-CD45RA, anti-CD45RO, anti-CCR7, anti-CD25, and anti-CD127 mAbs (BD Pharmingen). The expression of these molecules by T-cell lines was determined by flow cytometry (LSRII Fortessa, Becton Dickinson). Results are expressed as mean of ratio of fluorescence intensity (MRFI), representing the ratio between the mean of intensity of fluorescence of the stained sample with the selected mAb and that of the negative control (Maccalli C. *et al*, 2007), and as the % of positive cells. Raw data were elaborate with Kaluza software (IBeckman Coulter, instrumentation laboratories…).

5.4 IF and confocal microscopy analysis of CSCs.

CRC CSCs and FBS tumor cells were cultured (6 × 10^4 cells per well) onto cover glasses pre-coated with Matrigel in 24-well plates in culture medium, for 24 h. Cells were fixed onto glasses either with methanol for 10 min at −20°C or with 4% paraformaldeide for 10 min. at +4°C, washed with PBS, and then treated with PBS plus 0.1% Triton for 10 min. Afterward cells were incubated for 30 min. at 25°C with primary antibodies: anti-Survivin 8E2 (Thermo Scientific); and anti–COA-1 polyclonal antibody (Protein Expert) and Mage gp100 (Thermo Scientific). The bound antibodies were visualized with the Alexa Fluor-488–conjugated goat anti-mouse IgG F(ab')2 fragment (Molecular Probes). Nucleus were detected with Dapi (Invitrogen), samples were incubated for 10 min. Glasses were extensively rinsed with PBS and then mounted on microscope slides with FluorSave mounting solution (Millipore). Leica Confocal Microscopy analysis was done and multiple cells were analyzed for each staining condition with ×62, magnification.

5.5 NOD/SCID CB17 mice.

SCID mice are important tools for research cancer and vaccine development *in vivo*. So called because of their severe combined immunodeficiency, SCID mice have reduced ability to reject allogeneic or xenogeneic tissue grafts, and are therefore an excellent hosts for growing human tumor cells. The common denominator of all SCID mice is a spontaneous loss-of-function mutation of the protein kinase, DNA activated, catalytic polypeptide (*Prkdc*) gene. The SCID mutation was first identified in a BALB/c clonogenic strain (C.B-17), and it has been

bred onto a variety of different genetic backgrounds. As part of their nomenclature, all SCID mice contain the allele symbol "*Prkdcscid*". *Prkdc* functions in double-stranded DNA break repair and in recombining the variable (V), diversity (D), and joining (J) segments of immunoglobulin and T cell receptor genes. Because SCID mice cannot complete V(D)J gene recombination, their T and B cells do not mature and therefore cannot develop cell mediated and humoral adaptive immune responses. All Nod *Scid* CB17 mice used in this work were obtained from Charles River Laboratories and maintained under standard housing conditions in a pathogen-free environment, in the animal research facility of San Raffaele Institute.

5.5.1 Xenotransplants in NOD/SCID mice.

CSCs tumorigenicity was determined by injecting either CRC-derived CSCs or FBS tumor cells subcutaneously (s.c.) into adult (8-week old) NOD/SCID CB-17 mice. 100 microliters of a 1×10^5 cells suspension in physiological solution mixed at 1:1 ratio with matrigel containing medium (DMEM with 1% penicillin/streptomycin) were delivered by injection through a insuline syringe. Tumours formation was weekly monitored by a calliper and tumor volumes were estimated by using the following formula: ½ x (a x b^2), a= smaller diameter and b= larger diameter. When tumour masses reached 1-1,5 cm of diameter, mice were sacrificed (usually between 5 to 7 week following the subcutaneously transplantation, depending on the CSCs line under analysis). Tumor specimens were partly mechanically processed and the cell suspensions were cultured *in vitro* as previously described and partly processed for histologic and immunohistochemical examination. Statistical analysis of

differences between tumor volumes was done using two-tailed t test ($P < 0.05$).

5.5.1.1 CSCs serial dilutions in NOD/SCID mice.

100 microliters of a 1×10^5, 1×10^4, 1×10^3 and 1×10^2 cells suspension in physiological solution mixed at 1:1 ratio with matrigel containing medium (DMEM with 1% penicillin/streptomycin) were delivered by injection through a insuline syringe. We used three mice for each group. Tumours formation was weekly monitored by a calliper and tumor volumes were estimated by using the following formula: ½ x (a x b^2), a= smaller diameter and b= larger diameter. When tumour masses reached 1-1,5 cm of diameter, mice were sacrificed (usually between 5 to 7 week following the subcutaneously transplantation, depending on the CSCs line under analysis). Statistical analysis of differences between tumor volumes was done using two-tailed t test ($P < 0.05$).

5.5.1.2 CSCs serial transplantation in NOD/SCID mice.

Xenografts obtained by the subcutaneously injections of CRC-derived CSCs or FBS tumor cells (1×10^5 cells) were mechanically processed and the cell suspensions were propagated in the form of spheres in the specific growth medium (DMEM-F12 containing or not growth factors and RPMI 1640 supplemented with 10% FBS) for up 4-5 weeks. CSCs and FBS tumour cells obtained from derived primary xenografts were subsequently transplanted into secondary mice ($1x10^5$ cells). We used three mice for each conditions. To assess whether CRC CSC displayed long term tumorigenic potential the tumour volumes were monitored weekly by calliper

and compared with those of the first transplantation. Statistical analysis of differences between tumor volumes was done using two-tailed t test ($P < 0.05$).

5.6 Immunohistochemical analysis of xenografts tissues.

Xenografts obtained form the injection of CRC CSC and FBS tumor cells (patients #1076 and 1247) were fixed in 4% paraformaldeide O.N., submitted to a growing sucrose gradient (10%, 15% and 30%) and then embedded in OCT compound (Tissue-Tech) in presence of dry ice for 1 hour. Immunohistochemistry was done on 16-µm tissue sections, obtained with a cryostat. Sections were hydrated with PBS 1x and then stained with hematoxilyn and eosin for 30 seconds each. Sections were then dehydrated by dipping the glasses into a growing alcohol gradient solutions (alcohol 75%, 90%, 100% and xilene, respectively). Multiple sections of each sample were with ×4, ×10, ×20, and ×40 magnifications at microscopy.

Alternatively the 16-µm tissue sections, obtained with a cryostat, were incubated for 30 min. at 25°C with primary antibodies: anti-EpCAM sc-51681 (Santa Cruz Biotechnology, Inc), anti-HCAM 2C5 (R&D Systems, Inc.), anti-CD24 528807 (R&D Systems, Inc.) and anti-SOX2 MAB2018 (R&D Systems, Inc.). The bound antibodies were visualized with the Alexa Fluor-488–conjugated goat anti-mouse IgG F(ab')2 fragment (Molecular Probes). Nucleus were detected with Dapi (Invitrogen), samples were incubated for 10 min. Glasses were extensively rinsed with PBS and then mounted on microscope slides with FluorSave mounting solution (Millipore). Leica Confocal Microscopy analysis was done and

multiple cells were analyzed for each staining condition with ×62, magnification.

5.7 Detection of Prostaglandin-E2 (PGE2) release by CRC cells.

CRC CSC and FBS tumor cells (1.5×10^5 cells/ml) were plated in T25 flasks for 72h in DMEM without grow factors or FBS . The cell cultures were spinned at 2000 rpm for 10 min and the supernatants (0,5 ml) of each sample were used to determine the levels (pg/ml) of PGE2 released by using Prostaglandin E2 Expression EIA Kit (Cayman Chemical). Results were represented as averages of triplicates with an SD of ≤10%.

5.8 Isolation of peripheral blood mononuclear cells (PBMC) from CRC patients.

Peripheral blood was obtained from patients with a diagnosis of primary CRC admitted for surgery at the San Raffaele Hospital, Milan, Italy. About 30 ml of peripheral blood from each patients were placed in a tube containing 10 ml of Ficoll-Paque™ PLUS (GE Helthcare) and spinned at 800 rcf for 16 min. (brake off). Resultant layers were approximately from top to bottom: plasma, ring of PBMC, Ficoll and red blood cells (with granulocytes). The ring of PBMC was carefully aspirated and transferred to one new tube with PBS and spinned at 1700 rpm for 10 min. The pellet was incubated 10 min at +4°C with 5 ml of ACK Lysing Buffer (Invitrogen) and then spinned at 900 rpm for 10 min. PBMCs were resuspended in fresh medium and then counted with a Burker chamber

(we counted about 1-2x 10^6 lymphocytes per ml of whole blood).

5.9 Isolation of CRC-reactive T lymphocytes.

PBMCs from the peripheral blood of CRC patients were cultured *in vitro* with 100 IU/mL of rh-IL-2 (Chiron Corp.) and 10 ng/mL rh-IL-7 (PEPROTECH) in X-VIVO-15 (Cambrex Corp., Lonza) plus 10% human serum in the presence of autologous irradiated (200 Gy) CRC CSCs and FBS tumor cells (mixed lymphocyte tumor cell culture, MLTCs) pre-treated or not with INF-γ 1000IU/ml (PEPROTECH) for 48h. In some cases, PBMCs *in vitro* stimulated with autologous CRC CSCs or FBS tumor cells were cultured with rh-IL-2 plus 10 ng/mL of rh-IL-15 or of rh-IL-21 (PEPROTECH). In the case of #1247 MLTC the cultures were performed or not in the presence of 10 μg/ml of neutralizing Abs against IL-4 3007 (R&D Systems). Cell cultures were weekly stimulated with irradiated autologous CRC CSCs or FBS tumor cells; starting from the 3-4 weeks of culture, the reactivity of the T lymphocytes against CRC CSC or FBS tumor cell lines was determined by IFN-γ secretion measured by ELISPOT assay (Mabtech) following the previously described protocol by Maccalli *et al*., (2008). The T cells (5×10^3 cells per well) were incubated in flat-bottomed 96-well plates (Millipore, MultiScree 96-well plates) with integral filters, a plastic underdrain to prevent cross contamination between wells, and a plastic top cover, pre-coated with the anti-IFN-γ mAb in the presence of 1.7×10^4 cells per well of CRC cell lines. After 24 h of incubation at 37°C, cells were removed by emptying the plate and the plate was washed with PBS. The detection antibody (7-B6-1-biotin) was diluted at 1 μg/ml in PBS containing 0.5% fetal calf

serum and added 100 µl/well and incubated for 2 hours at room temperature. After washing the plate with PBS, the Streptavidin-ALP diluted 1:1000 in PBS-0.5% FCS was added 100 µl/well and incubate for 1 hour at room temperature. Then 100 µl/well of substrate solution (e.g. BCIP/NBT) was added and developed until distinct spots emerged. Colour development was stopped by washing extensively in tap water. The plate was left to dry. Spots were counted in an ELISpot reader.

The specificity of T lymphocyte recognition was assessed by the inhibition of IFN-γ secretion after the pre-incubation of the target cells with 10 µg/mL each of the anti-HLA class I mAb W6/32 and the anti-HLA class II (DR) mAb L243 (BD Pharmingen). T lymphocytes incubated with the mitogens phytohemagglutinin (PHA) served as positive controls. T lymphocytes without any stimulation represented the negative control. Statistical analysis of differences between means for cytokine release assays was done using two-tailed t test ($P < 0.05$).

Aims

The main aims of our study were to isolate cells with “tumor initiating properties” defined as CSCs from CRC tissues, and to performe their immunobiological characterization in order to assess whether these cells can be targeted by immunotherapy protocols.

6. Results

6.1 CSCs Isolation from human CRC tumors.

CRC CSCs have been isolated and established *in vitro* by the mechanical dissociation of tumor specimens deriving from patients admitted to the San Raffaele Hospital (Milan, Italy) for surgical resection of CRC (patients #1247, 1076, 11011, 10595, and 14583). Tumor cell suspensions were cultured in tissue culture 6-well plate at clonal density (2,500-5,000 cells/cm^2) in DMEM/F12 medium containing (growth factor dependent, GFD cells) or not (growth factor independent, GFI, cells) 50 ng/ml of EGF and 20 ng/ml b-FGF-2 (Figure 9).

We used the sphere formation (colon-spheres) approach to select CSC cultures that were then dissociated and re-plated to generate secondary cell cultures (Figure 9).

When enough tumor tissue was available, part of the cell suspensions obtained after dissociation were plated in the presence of RPMI 1640 supplemented with 10% FBS in order to select the differentiated plastic adherent cell components of CRC (Maccalli *et al*, 2003). These established cell lines, defined hereafter as FBS tumor cells, were used in parallel with CSCs for all the experiments, representing their non-CSCs pair lines.

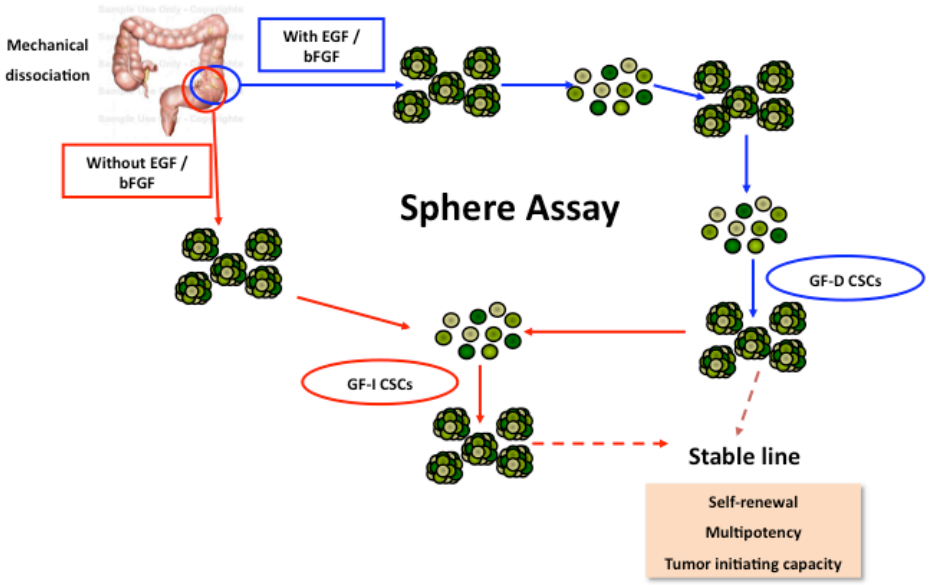


Fig.9 *Schematic representation of cancer stem cell sphere assay.* Briefly, CRC CSCs were isolated *in vitro* by mechanical dissociation of tumor specimens. Tumor cells suspensions were plated in tissue culture 6-well plates at clonal density (2,500-5,000 cells/cm^2) in DMEM/F12 medium containing (GFD) or not (GFI) 50 ng/ml of EGF and 20 ng/ml b-FGF-2, until they began to grow in the form of spheres.

6.2 Multipotency properties of CRC CSCs.

In order to verify the multi-lineage differentiation of our CSCs isolated *in vitro,* 1076 CSCs were cultured with medium containing FBS, representing a differentiating agent, for up to 10 days.

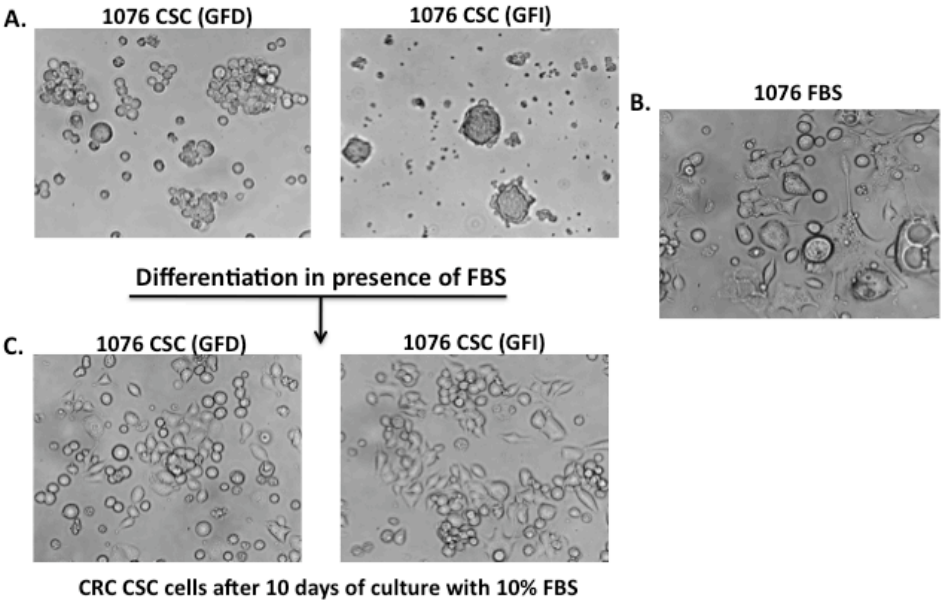


Fig.10 *CSCs multipotency properties assay*. 1076 CSCs (Panel A) were cultured with medium containing FBS, representing a differentiating agent, for up 10 days. At the end, the cells phenotype (Panel C) was compared with that of FBS tumor cells more differentiated phenotype (Panel B).

After this period of time, as the representative results of the cell lines isolated from patient #1076 show in Figure 10, cells have lost the ability to grow in suspension (Panel A) in the form of spheres and display adherence dependent growth (Panel C); thus showing a differentiated morphology similar to that of FBS tumor cells (Panel B). These results

indicated that our CSCs possess the fundamental stem-cell criteria of multipotency. Similar results have been obtained for the CSCs isolated from patients #1247 and #11011.

6.3 Expression of CRC- and CSC-associated molecules in both CSC and FBS cell lines.

To better characterize CRC CSCs and FBS tumor cells, the expression of normal and/or CSC-associated molecules was determined by Immunofluorescence (IF) and cytofluorimetric analysis (Figure 11). We focused our attention on the following markers: the epithelial cell adhesion molecule (EpCAM), the homing cell adhesion molecule (HCAM), the anchored surface molecule CD24, the carcinoembryonic antigen (CEA), the glycoprotein CD133, the levels of aldehyde dehydrogenase 1 (Aldefluor) and the transcription factors Nanog, Oct-4, Sox2.

Both CSCs and FBS tumor cells (#1247, 1076 and 11011) were positive for EpCam (3-6 MRFI), HCAM (2-6 MRFI), CD24 (2-6 MRFI), Aldefluor (2-9 MRFI), CEA (2-9 MRFI) and Sox-2 (2-8 MRFI) (Figure 3). Oct-4 was not expressed in all these cell lines with the exception of 1247 FBS (MRFI 2), while Nanog was expressed at low levels in #1247 and #1076 deriving cell lines (both CSC and FBS) (2-3 MRFI) but not found in #11011 cell lines. CD133 was expressed at low levels or negative both in CSC and FBS tumor lines. In addition, the 1247 CSC lines expressed higher levels of EpCAM, CD24 and HCAM (2 and 6 fold increase), than the corresponding 1247 FBS cell lines (Figure 11). On the contrary, 1076 CSC GFD showed higher levels of CD24 (2 fold increase), CEA (1.6 fold increase), and Sox-2 (2.5 fold increase) than the corresponding 1076 FBS cell lines

No significant differences of MRFI of the analyzed markers were detectable for 11011 CSC compared to the 11011 FBS counterpart, with the only exception for CEA, which expression was 2.3 times higher.

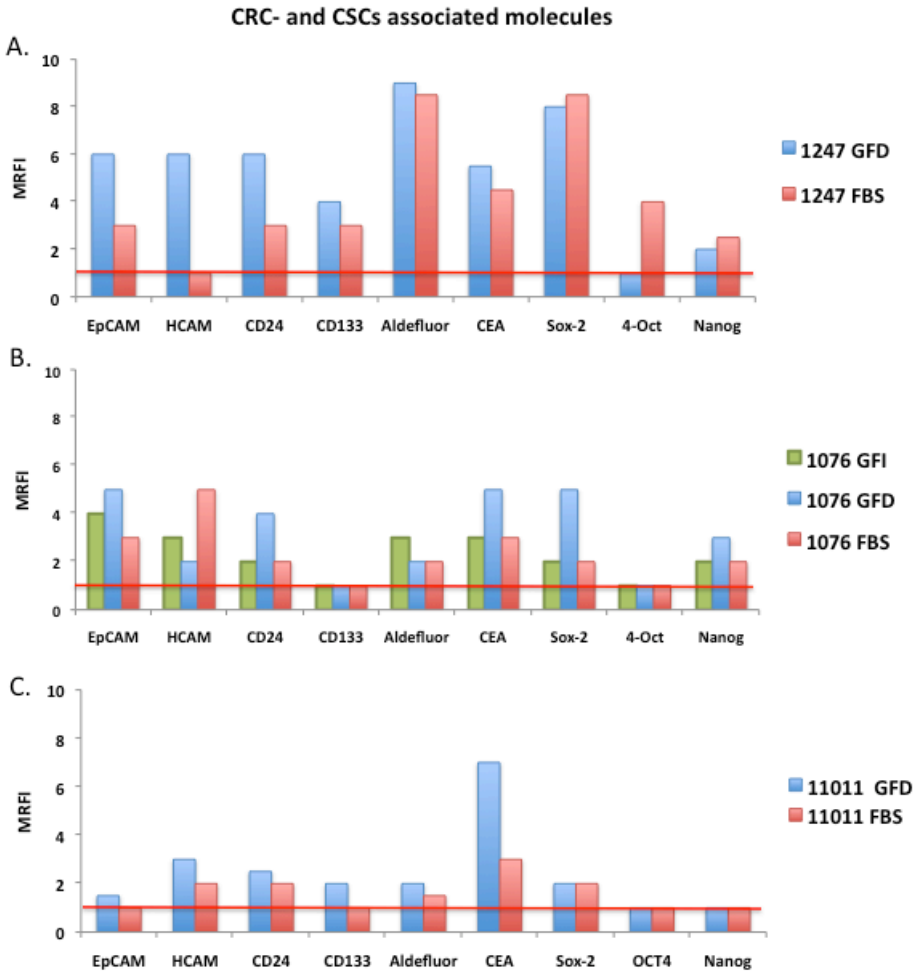


Fig.11 *CRC- and CSC-associated molecules expression by CSC vs. FBS tumor cells.* The expression of CRC and FBS-associated molecules was evaluated by IF, using specific mAbs, and cytofluorimetric analysis. The results are represented as MRFI (Relative Mean of Flourescence Intensity) that is the ratio between the mean of florescence intensity of cells stained with the selected mAb and that of the negative control. Significant value were considered ≥ 2 MRFI.

6.4 Tumorigenicity of CSC lines injected in NOD/SCID mice.

The tumorigenic potential of the CSC and their FBS tumor cell pairs isolated from CRC patients #1247 and 1076 was determined by injecting the cells subcutaneously into adult NOD/SCID mice (as described in Materials and Methods).

$1x10^5$ cells (both CRC CSC and FBS) tumour-deriving cells were subcutaneously injected into immunodeficient NOD/SCID CB17 mice (8-week old female) and the formation of tumours was evaluated weekly by a calliper (Figure 12). CSCs grew more efficiently than FBS.

Patient #1247 generated visible tumours after 1-2 weeks from the transplant, notably #1247 CSCs grew more efficiently than #1247 FBS tumour cells: larger tumour volumes were obtained since the fourth week (1.5 cm^3 1247 CSC vs. 0.8 cm^3 1247 FBS) and after only 5 weeks #1247 CSCs gave rise to 2 mm^3 tumour mass vs. 1.2 cm^3 tumour mass generated by 1247 FBS. At this time point mice were sacrificed (Panel A). Differently, patient #1076 generated visible tumours after 2-3 weeks from the transplant, and also in this experiment both 1076 CSC GFD and #1076 CSC GFI grew more efficiently than 1247 FBS tumour cells.

Larger tumour volumes were detectable since the sixth week (1.07 cm^3 1076 CSC GFD, 1.2 cm^3 1076 CSC GFI *vs*. 0.6 cm^3 1076 FBS). Notably 1076 CSC GFD and 1076 CSC GFI gave rice similar volumes of tumour mass at the different time points and no significant differences were detectable between the two lines. After 7 weeks from the transplant 1076 CSC GFD and 1076 CSC GFI gave rice to 1.7 cm^3 and 1.8 cm^3 tumour masses respectively vs. 1076 FBS 0.9 cm^3 tumour mass, at this time point mice were sacrificed (Panel B). On the basis of these evidences we

can conclude that CRC CSC vs. FBS cell lines showed a more efficient capacity to initiate tumor growth when injected in nude mice.

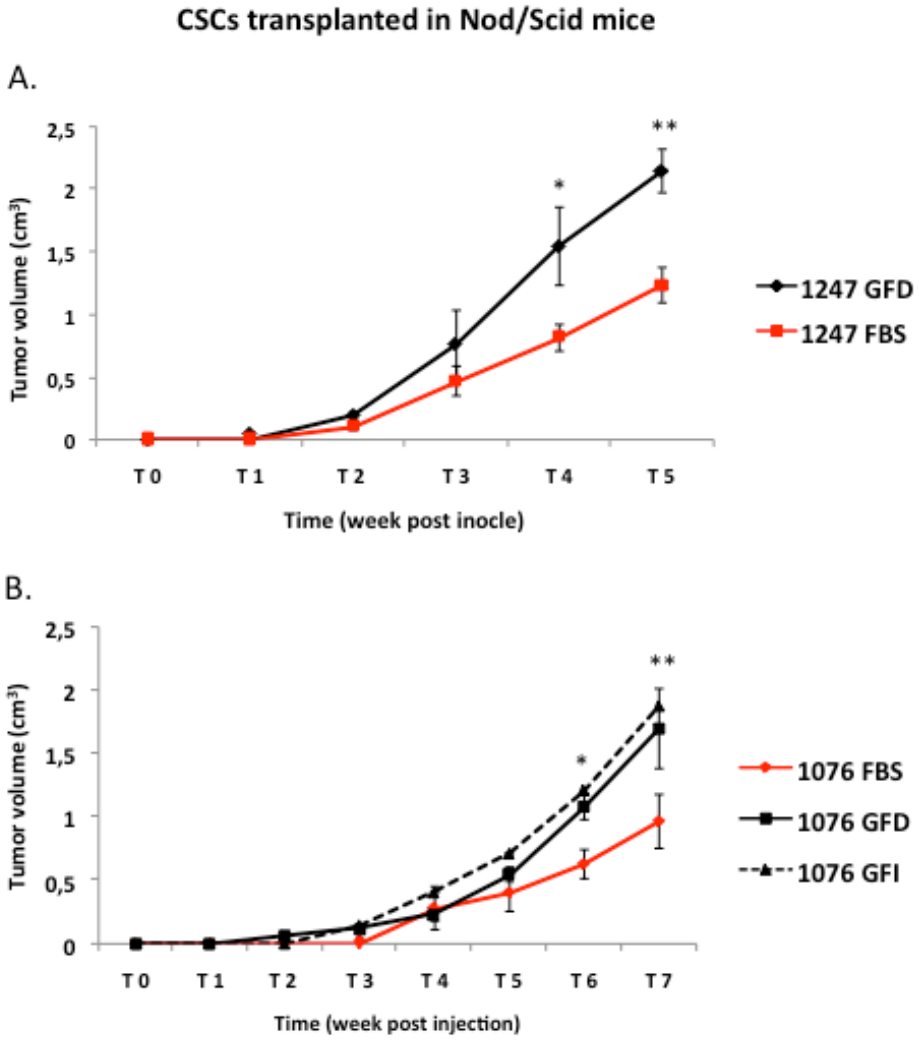


Fig. 12. *CSCs transplanted in NOD/SCID mice*. The tumorigenic ability of 1247 (Panel A) and 1076 (Panel B) CSC vs FBS lines was evaluated by the injection either CRC CSC or FBS tumour cells in NOD/SCID mice. $1x10^5$ cells were mixed at 1:1 ratio with matrigel containing medium (DMEM with 1%

penicillin/streptomycin), then the cells (100 µl volume) were subcutaneously (s.c.) injected into immunodeficient NOD/SCID CB17 mice (8-week old female), obtained from the Charles River Laboratories. Tumour formation was monitored weekly and when tumour masses reached the volume 1-1,5 cm^3, mice were sacrificed. Data concerning the tumour volumes generated by injected cells are mean ± s.d. of two independent experiments in triplicate. ∗: means $p < 0.05$ (95% t-TEST) and ∗∗: means $p < 0.01$ (99% t-TEST).

6.5 Immunohistochemical analysis of xenograft generated tumors.

A preliminary morphological analysis of CRC CSC and FBS lines derived xenografts was performed by immunohistochemistry. Tissues obtained from the injection of CSC or FBS tumor cells in mice were stained with hematoxilyn and eosin (H&E). The morphological analysis of tumour sections revealed that CSC-derived tissues showed larger areas of necrosis, (see the black arrows) than the tissues generated by FBS tumor cells (Figure 13). In addition, #1076 CSC GFD-derived tissues showed some areas of vasculature, as indicated by the red arrows (Panel D). This morphological phenotype is likely to be due to the higher proliferative potential of CRC CSC.

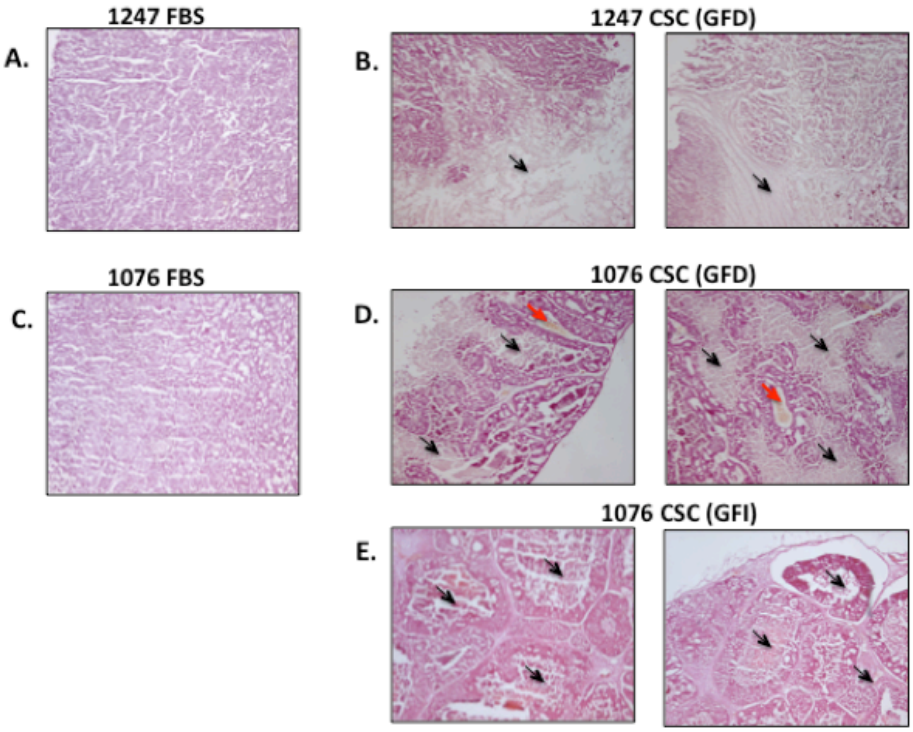


Fig.13. *Immunohistochemical analysis of tumor tissues deriving from xenograft generated tumors in NOD/SCID mice*. 1247 FBS (panel A), 1247 CSC GFD (panel B), 1076 FBS (panel C), 1076 CSC GFD (panel D) and 1076 CSC GFI (panel E). 16 μm tissue sections were stained with hematoxilyn and eosin (H&E); 10x magnification are shown in the figure. Black arrows point the areas of necrosis while red arrows point areas of vasculature.

6.6 Expression of CSC-associated markers by #1247 CRC-derived xenograft tissues.

The expression of some markers associated to CRC and /or CSCs, such as HCAM, EpCam, CD24 and Sox-2, was determined by IF analysis of CRC primary tissues, and CSCs and FBS cell line-deriving xenograft tumors. Representative results of tissue and cell line xenograft deriving tumors from patient #1247 are shown in Figure 14 1247 CSC xenografts (Panel B) and the parental tumor (Panel A) exhibited similar patterns of expression of Hcam, EpCam and Sox-2, while CD24 was expressed at higher levels by 1247 CSC xenografts. The xenograft tumors generated by 1247 FBS (Panel C) cell line expressed lower level of these markers than xenografts generated by #1247 CSCs (Panel B). Thus, the xenografts generated in this model matched the phenotype of the original tumor.

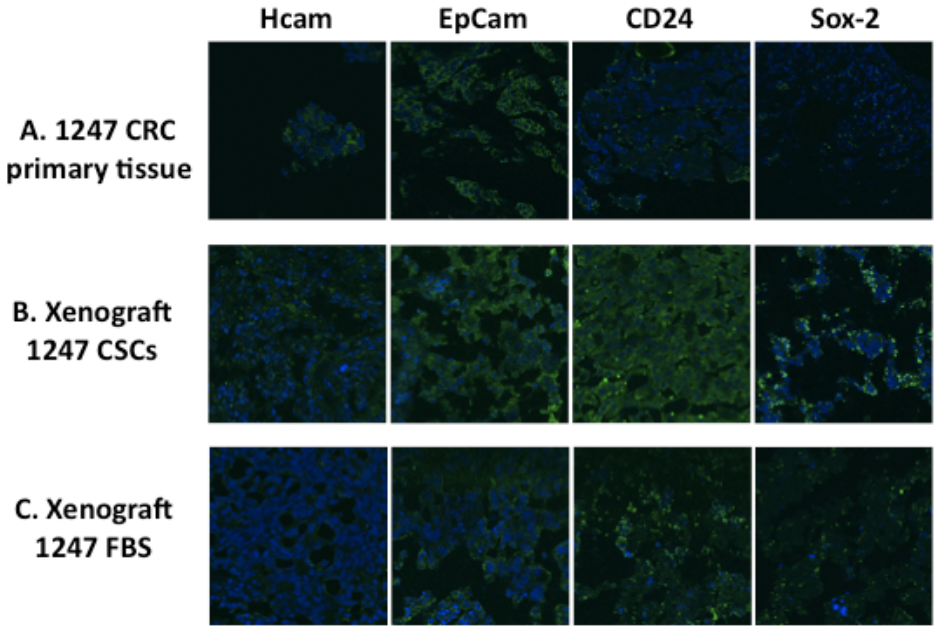


Fig. 14 *Expression of CRC- and/or CSC-associated molecules* by the 1247 primary tumor (Panel A) and xenograft deriving tissues: 1247 CSCs (Panel B) and 1247 FBS (Panel C). HCAM, EpCam, CD24 and Sox-2 expression was determined by IHC analysis using specific mAb of tumor tissues. Goat-anti-mouse FITC or Alexa488 conjugated was used as secondary staining Ab. Imagines were obtained with a Leica Confocal microscopy, 62x magnification.

6.7 CSC serial dilution and serial transplantation in NOD/SCID mice.

To determine whether differences in the tumorigenic potential of CSC vs. FBS tumour cells could occur we have performed serial dilution experiments in the xenotransplant system (Figure 15).

Serial cell dilutions ($1x10^5$, $1x10^4$, $1x10^3$ and $1x10^2$ cells) for each cell lines were injected into NOD/SCID mice and the tumour formation has been weekly monitored (representative results of patient #1247 are shown in Figure 15).

Serial dilutions from #1247 CSCs and 1247 FBS xenografts displayed an exponential growth that had the propensity to increase with the serial cell concentration. We could observed that #1247 CSCs could develop, tumours *in vivo* also by the injection of low cell concentrations (Panel A). Notably #1247 CSCs were able to generate tumours with higher efficiency compared to #1247 FBS cells, by the injection of only $1x10^3$ cells. Visible tumours began to be detectable two weeks later (time point 3) than those appearing after the high concentration cells injection (0.7 cm^3 1247 CSCs $1x10^5$ cells vs. 0.05 cm^3 1247 CSCs $1x10^3$ cells). After 5 weeks the differences in tumour volumes between the different cells concentrations was also detectable and significant 2.1 cm^3 1247 CSCs $1x10^5$ cells, 1.5 cm^3 1247 CSCs $1x10^4$ and 0.7 cm^3 1247 CSCs $1x10^3$ cells, while the injection of $1x10^2$ 1247 CSCs failed to generate tumours.

Differently 1247 FBS tumour cells were able to generate tumours up to the injection of $1x10^4$ cells: visible tumours began to be detectable two weeks post the injection and significant differences in tumour volumes between the two cell concentrations began to be detectable at week 4: 0.8 cm^3 1247 FBS $1x10^5$ cells vs. 0.5 cm^3 1247 FBS $1x10^3$ cells. At week

5 the differences in tumour volumes were also detectable and statistically significant: 1.2 cm^3 1247 FBS $1x10^5$ cells, 0.8 cm^3 1247 FBS $1x10^4$, while the injection of $1x10^3$ 1247 FBS began to be detectable (0.1 cm^3 tumour volume) (Panel B).

On the basis of these data we can conclude that CRC CSCs vs. FBS cell lines showed a more efficient capacity to initiate tumour growth in nude mice at lower concentrations.

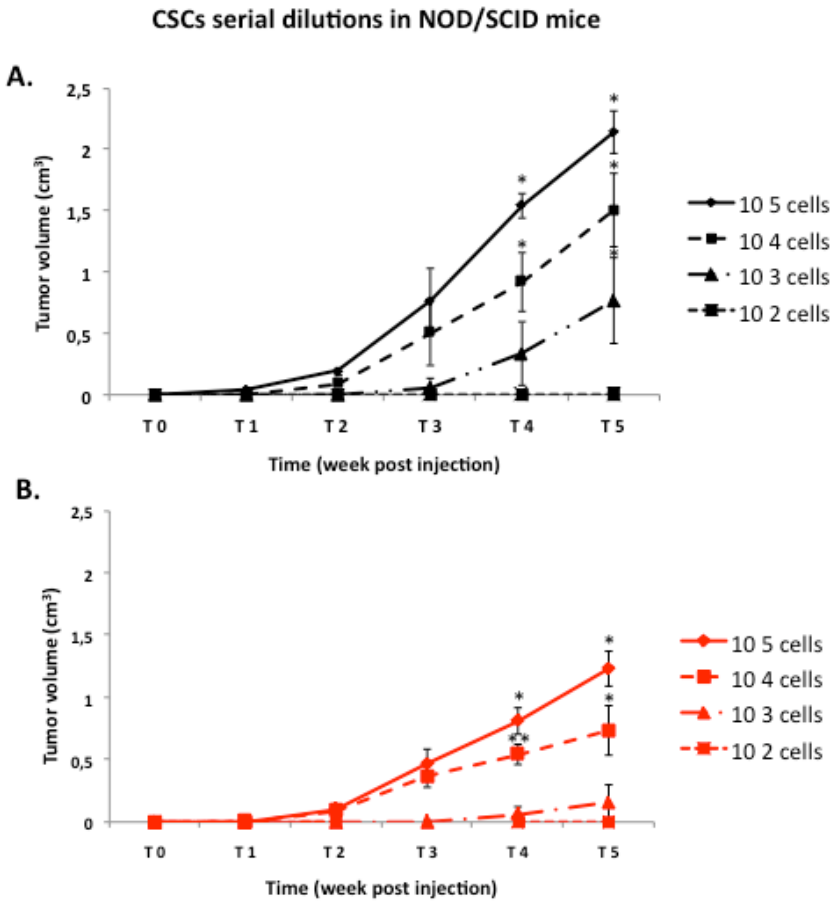


Fig.15 *Serial dilution of #1247 CSCs and FBS tumour cells.* $1x10^5$, $1x10^4$, $1x10^3$ and $1x10^2$ cells either of 1247 CRC CSC (panel A) or 1247 FBS (panel B) tumour cells were diluted at 1:1 ratio in matrigel containing medium (DMEM with 1% penicillin/streptomycin) and were subcutaneously (s.c.) injected (100 µl of final volume) into immunodeficient NOD/SCID CB17 mice (8-week old female), obtained from the Charles River Laboratories. Tumour formation was monitored weekly and when tumour masses reached the volume of 1-1.5 cm^3, the mice were sacrificed. Data are indicated as the means ± s.d. of tumour volumes deriving from two independent experiments. Three mice each treatment were used for every single experiment. ∗ : means $p < 0.05$ (95% T-TEST) and ∗∗: means $p < 0.01$ (99% T-TEST).

To assess whether CRC CSCs displayed long term tumorigenic potential, we evaluated the ability of these cells to generate tumours after serial transplantations. Tumour xenograft derived by the injection of $1x10^5$ cells (#1247 CSC and 1247 FBS are shown in Figure 16) were mechanically processed for *in vitro* culture. After 4 weeks of *in vitro* culture, CSC and FBS tumour cells, obtained from primary xenograft tissues, were transplanted into new mice (Panel B). Tumour formation was monitored weekly. Data confirmed the ability of #1247 CSCs to generate tumours into secondary mice as well, with a tumour volume resembling that of the initial transplantation (Panel A). On the contrary, 1247 FBS cells following the second injection in mice gave rise to tumours with reduced dimensions compared with the CSC-derived tissues (1.2 vs. 0.9 cm^3).

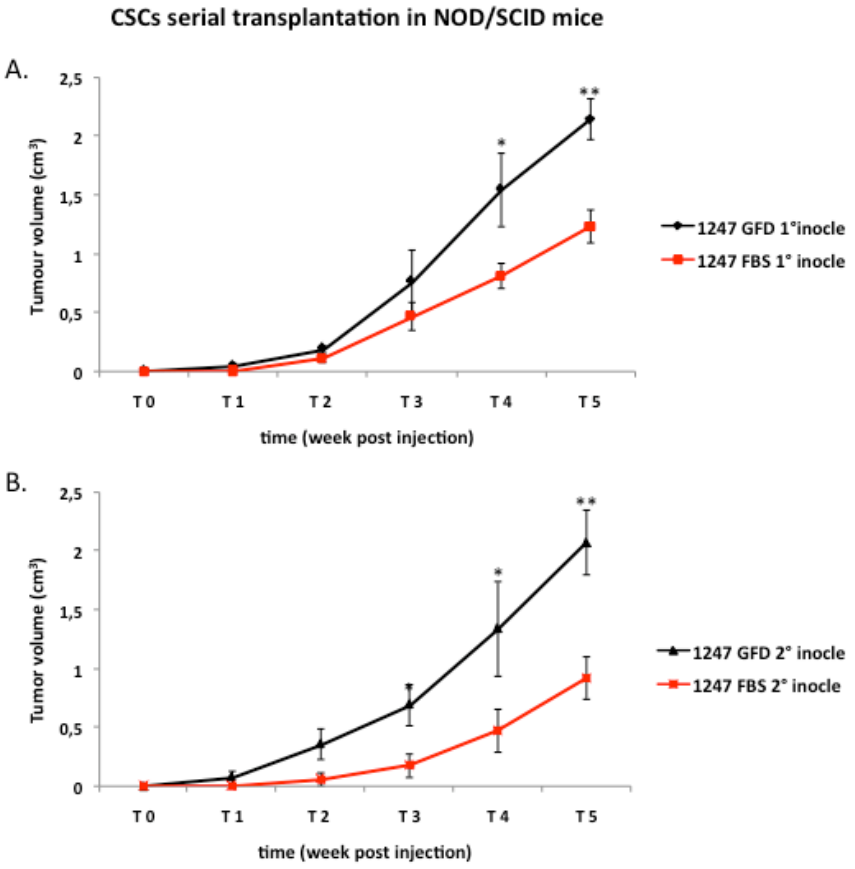


Fig.16. #1247 *CSCs and FBS tumour cells serial transplantation in NOD/SCID mice.* Either CRC CSCs or FBS tumour cells ($1x10^5$ cells) were mixed at 1:1 ratio with matrigel containing medium (DMEM with 1% penicillin/streptomycin), then the cells (100 µl volume) were subcutaneously (s.c.) injected into NOD/SCID CB17 immunodeficient mice (8-week old female), obtained from the Charles River Laboratories (Panel A). After 4-5 weeks, visible tumours arose (1-1.5 cm^3 tumour sizes) then mice were sacrificed and tumour samples were removed and mechanically processed for *in vitro* culture. CSCs and FBS tumour cells obtained from derived primary xenografts were subsequently transplanted into secondary mice ($1x10^5$ cells) (Panel B) and tumour formation was monitored weekly. Data concerning the tumour volumes generated by injected cells are mean ± s.d. of two independent experiments in triplicate. ∗: means $p < 0.05$ (95% T-TEST) and ∗∗: means $p < 0.01$ (99% T-TEST).

6.8 Expression of MHC molecules and NKG2D ligands by CSCs vs. FBS tumour cells.

The expression of MHC class I and class II molecules by CRC CSCs and their FBS tumor pairs was evaluated by immunofluorescence and cytofluorimetric analysis. Figure 17 shows the results of two representative primary CSC lines #1247 and #1076. MHC class I molecules were detectable both in CSCs and FBS tumour cells, although at relatively low levels (2-4 MRFI). MHC class II expression was negative in all the analyzed cell lines.

The NKG2DLs expression by primary CSC lines 1247 and 1076 was quite homogeneous (80-90%) (data not shown).

Figure 17 shows the MRFI of NKG2DLs by CSC and their FBS tumour cell pairs isolated from patient #1247 and #1076 (Panels A and B, respectively). Notably 1247 CSCs expressed higher values of MRFI than the 1247 FBS: MICA (2 fold increase), MICB (2.5 fold increase), ULBP-1 (4.6 fold increase), ULBP-2 (2.5 fold increase), ULBP-3 (4 fold increase), and ULBP-4 (6.3 fold increase).

On the contrary, in patient #1076, ULBP-1 (7 MRFI), ULBP-2 (2 MRFI), and ULBP-3 (4 MRFI) were expressed at the same level by 1076 CSC GFD, 1076 CSC GFI and 1076 FBS while MIC-A and ULBP-4 were slightly up-regulated by 1076 CSC GFI (1.5 and 1.25 fold increase, respectively) than 1076 FBS. At last MIC-B was the only molecule up-regulated in both 1076 CSC GFD (2 fold increase) and 1076 CSC GFI (2.5 fold increase) than 1076 FBS.

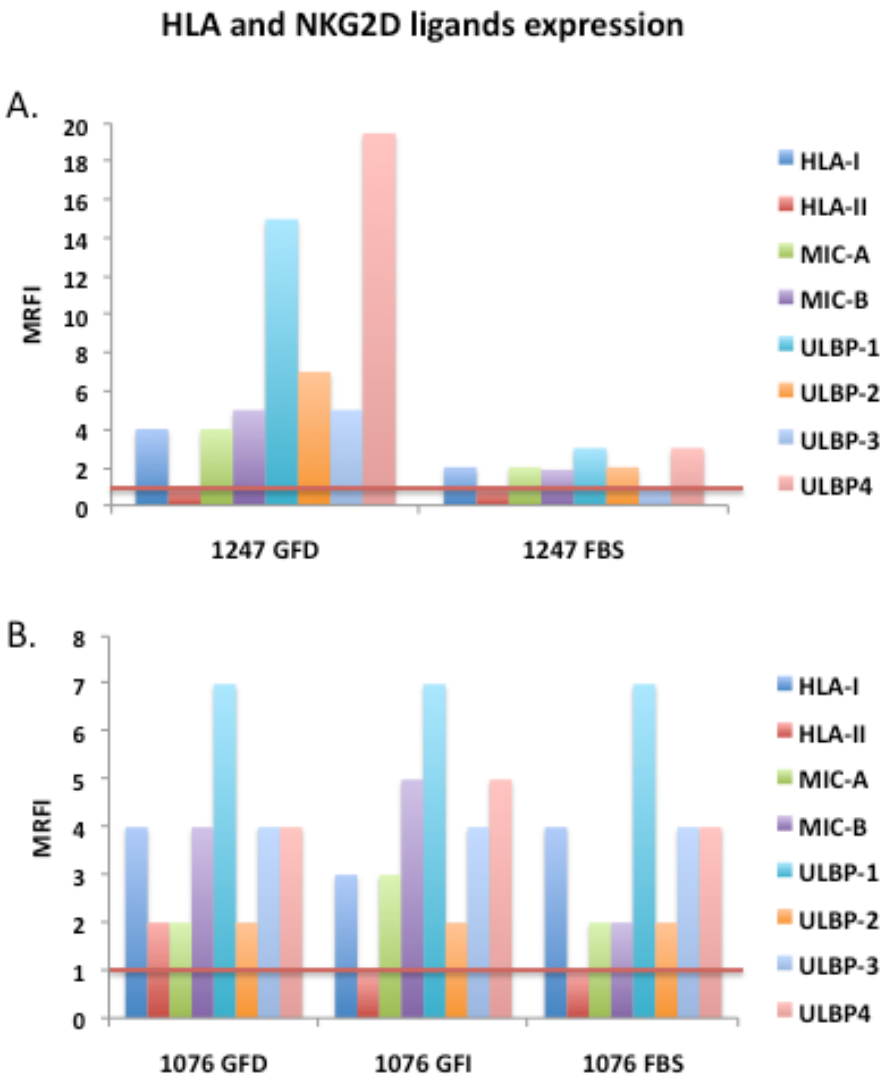


Fig.17 *Expression of MHC class I and II and molecules and NKG2DLs by CSCs vs. FBS cell lines.* The expression of MHC molecules and NKG2DLs was determined by Immunofluorescence (IF), using specific mAbs and cytofluorimetric analysis. Data are represented as MRFI (Relative Mean of Flourescence Intensity) that is the ratio between the mean of flourescence intensity of cells stained with the selected mAb and that of the negative control. Significant value were considered ≥ 2 MRFI.

6.9 Antigen Processing Machinery (APM) molecules expressed by CSC and FBS tumor cells.

The expression of a large number of molecules belonging to the APM was examined by intracellular staining and cytofluorimetric analysis in patients #1247,1076 and 11011. These molecules included MHC class I molecules and HLA class I heavy chains (i.e., A-HC), β2-microglobulin, constitutive proteasome subunits (delta, MB1, and Z), immunoproteasome (LMP2, LMP7, and LMP10), transporter molecules (TAP), and chaperon molecules (tapasin, calnexin, calreticulin, and ERp57) (Table 4).

Heterogeneous detection of the expression of HLA-A, HLA-B, and HLA-C molecules was observed by CRC CSCs and FBS tumour cell lines, with in most cases higher levels in FBS tumour cells than in CSCs (Table 4). Similar results were obtained for the staining of A-HC and the β2-microglobulin (Table 4).

Expression of immunoproteasome components LMP2 and LMP7 by both CRC CSCs and FBS tumour cells isolated from patient #1247 was commonly detected, although with heterogeneous values of MRFI (2-10 and 2-6, respectively). Low expression of LMP10 was observed in most lines and LMP10 was negative in 2 of 8 cell lines (1247 CSC GFD and 1076 CSC GFI) (Figure 18, panel A).

TAP1 and TAP2 were negative in 1247 CSC, 1076 CSC GFI while high levels of expression were observed in FBS tumour cells: 1247 FBS (2 and 5 MRFI, respectively) and 11011 FBS (3 and 8 MRFI, respectively) and in two CSC lines: 1076 CSC GFD (4 and 7 MRFI, respectively) and 11011 CSC (3 and 6 MRFI, respectively) (Figure 18, panel B).

#1247 CSC and 11011 CSC lacked or showed a low expression of the chaperon molecules ERp5, and Tapasin. At the same time these molecules were up-regulated (3 fold increase, respectively) in their #1247 and #11011 FBS counterpart (Figure 18, Panel C).

Calnexin was the only APM molecule widely (3-104 MRFI) detected in all CSC and FBS tumour cell lines.

In conclusion two CSC lines, #1247 CSC GFD and #1076 CSC GFI, showed low or negative levels of expression of a variety of APM molecules while these molecules were detectable in their FBS tumour cell pairs (Table 4), thus suggesting that defects in antigen processing and presentation can occur preferentially in CSCs.

Molecule	1247 GFD	1247 FBS	1076 GFD	1076 GFI	1076 FBS	11011 GFD	11011 FBS
HLA-A	2 (64)	**6** (100)	**3** (64)	2 (23)	**3** (26)	**18** (97)	**5** (87)
HLA-B	2 (68)	**4** (100)	**4** (68)	2 (26)	**4** (74)	**8** (97)	**4** (94)
HLA-C	2 (79)	**4** (100)	**7** (79)	2 (66)	**4** (68)	n.d.	n.d.
HLA-I hc (10)	2 (61)	**3** (96)	**3** (61)	1 (17)	**3** (49)	n.d.	n.d.
HLA-I hc (A2)	2 (62)	2 (99)	2 (62)	2 (24)	**3** (47)	n.d.	n.d.
HLA-I hc	**3** (95)	**11** (100)	**24** (95)	**3** (86)	**10** (98)	n.d.	n.d.
β2-micr. (KJ2)	2 (75)	**5** (100)	**16** (75)	2 (32)	**4** (80)	**35** (99)	**10** (98)
β2-micr. (L368)	2 (66)	**3** (100)	2 (66)	2 (16)	**3** (28)	n.d.	n.d.
β2-micr. (NAMB1)	**3** (89)	**6** (100)	**15** (89)	**3** (69)	**8** (96)	**76** (97)	**17** (99)
HLA-DR,DQ,DP	1 (43)	**3** (100)	**5** (43)	1 (31)	**3** (46)	**11** (99)	**14** (97)
delta	2 (93)	**11** (100)	**26** (93)	**3** (93)	**10** (99)	**82** (99)	**83** (98)
MIB-1	2 (80)	**6** (100)	**14** (80)	2 (53)	**4** (78)	**12** (97)	**11** (97)
Z	1 (39)	**3** (98)	**15** (39)	1 (18)	**3** (22)	**6** (99)	**5** (99)
LMP-2	2 (75)	**10** (100)	**4** (75)	2 (62)	**6** (94)	**29** (99)	**46** (99)
LMP-7	2 (69)	**6** (100)	**33** (69)	2 (66)	**4** (75)	**11** (99)	**14** (96)
LMP-10	1 (40)	**3** (100)	**13** (40)	1 (18)	**3 (**56)	**3** (96)	**5** (97)
TAP-1	1 (35)	2 (98)	**4** (35)	1 (19)	1 (31)	**3** (96)	**3** (98)
TAP-2	1 (59)	**5** (100)	**7** (59)	2 (35)	1 (57)	**6** (96)	**15** (98)
Calnexin	**3** (95)	**17** (100)	**104** (95)	**5** (95)	**6** (100)	**72** (98)	**101** (99)
Calreticulin	2 (77)	**3** (100)	**18** (77)	**4** (89)	**4** (89)	**60** (98)	**52** (99)
ERp57	1 (37)	**3** (99)	**3** (37)	1 (13)	1 (13)	**3** (96)	**9** (97)
Tapasin	1 (42)	**3** (100)	**7** (42)	**4** (24)	1 (24)	**3** (96)	**9** (94)

Table 2. *Expression of APM in CRC CSCs and FBS tumour cell lines*. The expression of the indicated molecules was evaluated by intracellular staining onto permeabilized cells with specific mAbs and by cytofluorimetric analysis; *see* Materials and Methods. Data are represented as MRFI that is the ratio between the mean of fluorescence intensity of cells stained with the selected mAb and that of the negative control; bold values indicate MRFI > 2. The numbers in parenthesis represent the percentage of positive cells. The experiment has been repeated three times; SD for each value < 5. n.d.= not done.

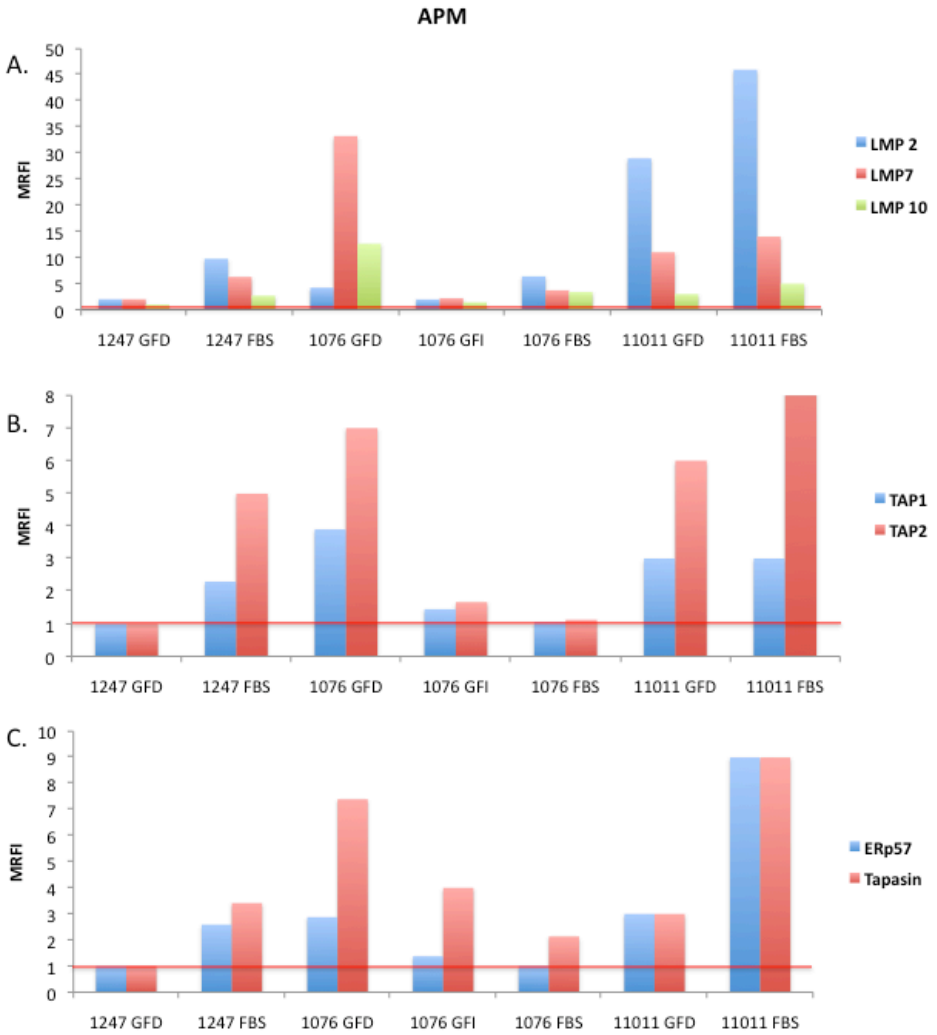


Fig.18 *Expression of APM molecules by CSCs vs. FBS tumor cells.* LMP2, LMP7 and LMP10 (panel A), TAP1, TAP2 (panel B), Erp57 and Tapasin (panel C) expression by by CSC vs. FBS cell lines was evaluated by Immunofluorescence (IF) and cytofluorimetric analysis. Data are represented as MRFI (Relative Mean of Flourescence Intensity) that is the ratio between the mean of flourescence intensity of cells stained with the selected mAb and that of the negative control. Significant value were considered ≥ 2 MRFI.

6.10 CSCs and FBS cell lines can express immune-regulatory molecules.

To assess the expression by #1247, 1076, 11011, 14583 and 10595 of immune regulatory molecules, such as CTLA-4 and its ligands B7-1 and 2, PD-1 and its ligands PDL-1 and PDL-2, B7H2 and B7H3 the phenotype analysis with the specific mAb and the cytofluorimetric analysis has been performed (Figure 19).

All CSCs and FBS cell lines were homogeneously positive for CTL-4, PD-1 and, although at higher levels, PD-L1 (Figure 19). CTL-4 expression was surprisingly found both in membrane and in the cytoplasm (Figure 19, Panel A).

These cell lines were negative for PD-L2 with the exception of two cell lines that were found to express low levels; 1247 GFI (2 MRFI) 14583 GFD (3 MRFI) (data not shown).

Furthermore, both B7-1 and B7-2 molecules that represent ligands of either the T cell–associated co-stimulatory molecule CD28 or the inhibitory CTLA-4 molecule were detected in only 2 of 9 lines (data not shown) 11011 FBS (5 and 3 MRFI, respectively) and 14583 GFD (3 and 5 MRFI, respectively).

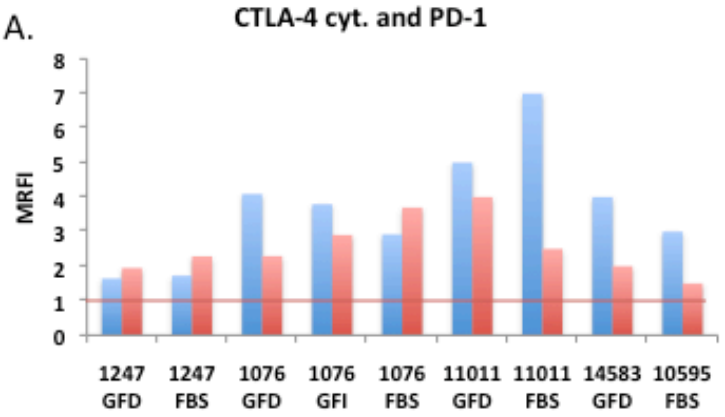
Thus, CRC CSCs and FBS tumour cells expressed significant levels of negative regulators of T-cell responses (e.g., CTLA-4 and PD-1) (Panel A) and their ligands (e.g. PD-L1) (Figure 19, Panel B).

We have also determined the expression of B7-H3, one of the most recently identified members of the B7/CD28 superfamily serving as an accessory negative modulator of T-cell responses. Notably B7-H3 expression by both CRC CSC and FBS tumour cells was very high (12-

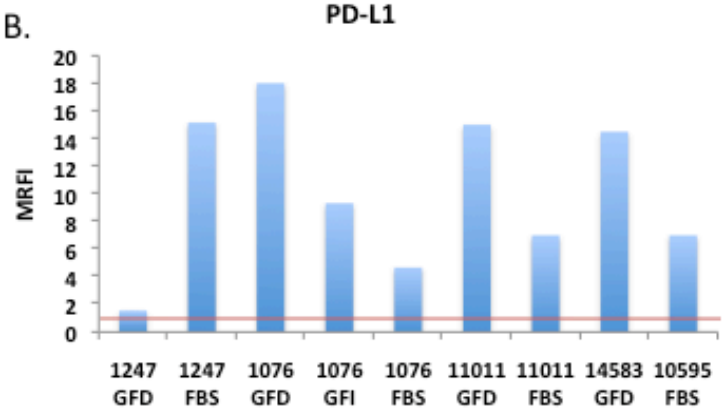
to 65-fold of MRFI) in 6 of 9 cell lines (Figure 19, Panel C).

Therefore, the low level of expression of APM components and/or NKG2DLs, the lack of the expression of co-stimulatory molecule ligands, such as B7-1 and B7-2, and the expression of immune regulatory molecules by CRC CSCs render these cells weakly immunogenic.

A.


CTLA-4 cyt. and PD-1
MRFI
0
1
2
3
4
5
6
7
8
1247 GFD
1247 FBS
1076 GFD
1076 GFI
1076 FBS
11011 GFD
11011 FBS
14583 GFD
10595 FBS

B.


PD-L1
MRFI
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1076 FBS
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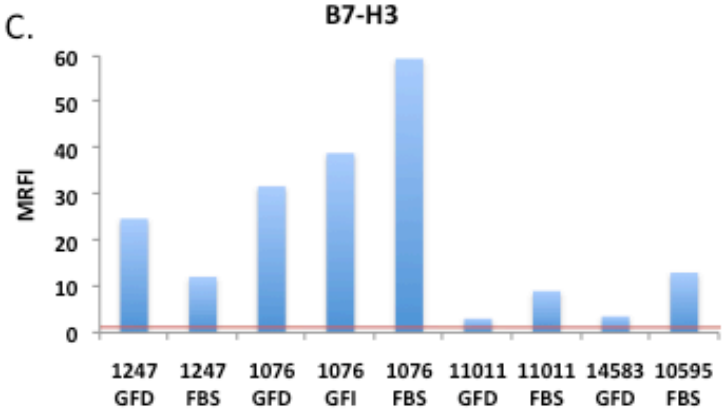
11011 FBS
14583 GFD
10595 FBS

C.


B7-H3
MRFI
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10
20
30
40
50
60
1247 GFD
1247 FBS
1076 GFD
1076 GFI
1076 FBS
11011 GFD
11011 FBS
14583 GFD
10595 FBS

Fig.19 Expression of some immune-regulatory molecules: CTLA-4 cytoplasmic staining (red bar) and PD-1 (blue bar) (panel A), PD-L1 (panel B) and B7-H3 (panel C) by CSC vs. FBS cell lines (#1247, 1076, 11011, 14583 and 10595), evaluated by IF and cytofluorimetric analysis. Data are represented as MRFI (Relative Mean of Flourescence Intensity) that is the ratio between the mean of fluorescence intensity of cells stained with the selected mAb and that of the negative control. Significant value were considered ≥ 2 MRFI.

6.11 Analysis of the expression of tumor associated antigens (TAAs) by CRC CSCs vs FBS cells.

The expression by CRC CSCs of a panel of some colorectal TAAs was analyzed by IF and microscopy analysis.

We focus our attention on: a) colon antigen-1 (COA-1), able to induce both $CD8^+$ and $CD4^+$ T-cell-mediated responses (Maccalli C. et al., 2008), b) Survivin, an inhibitor of apoptosis, whose over-expression was recently found to correlate with increased invasion and metastasis of colorectal cancer (Xiao-Yuann Chu et al., 2011) and c) on MAGE-A3, a melanoma TAA described in literature not to be expressed at high levels in CRC cancer.

Representative results of #1076-derived cell lines are shown in the Figure 20. Both CSC and FBS tumor cell lines homogenously displayed high levels of CRC associated antigen COA-1 and Survivin. 1076 CSC cell lines were negative for MAGE-A3, while only few 1076 FBS cells weakly expressed the antigen. Thus, the TAAs COA-1 and Survivin may represents candidate target molecules for T cell-mediated anti-CSCs reactivity.

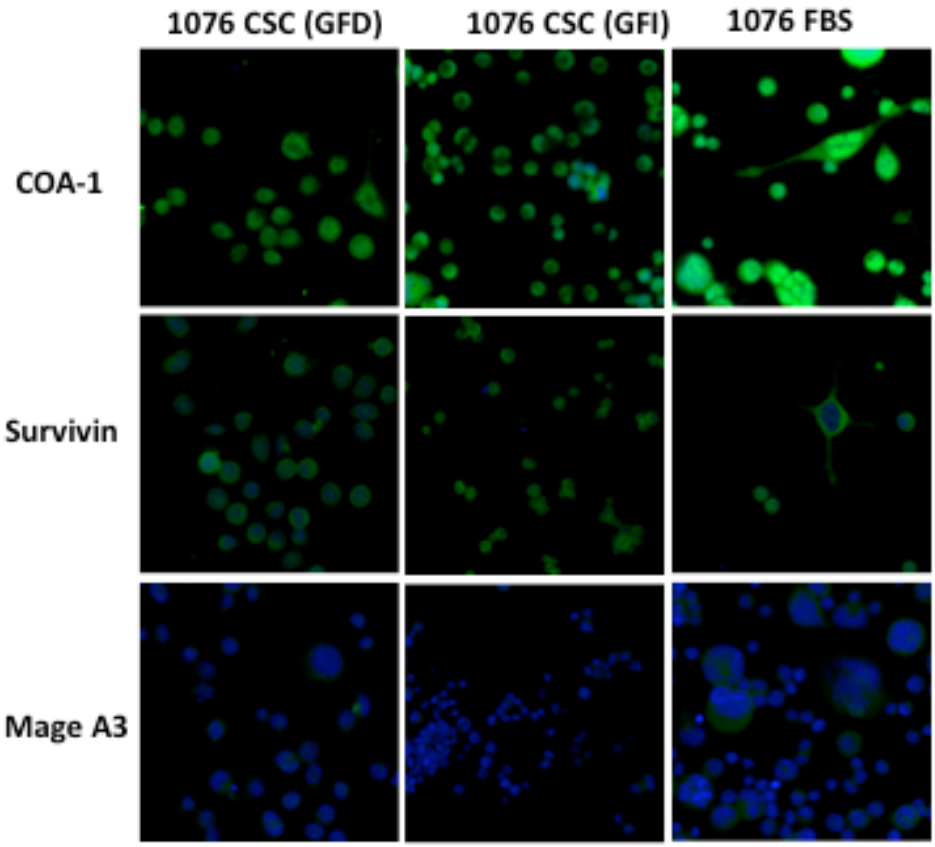


Fig.20 *Expression of a panel of TAAs by CSC vs. FBS tumor cells*. The expression of COA-1, Survivin, and MAGE-A3 was analyzed by IF with specific mAbs and goat-anti-mouse FITC or Alexa488 conjugated as secondary staining Ab and microscopy analysis. Representative results of 1076-derived cell lines are shown in the Figure. 40x magnification is shown.

6.12 Cytokines release by CRC CSCs vs FBS cells.

The expression of the cytokine IL-4 and its receptor IL-4R by CRC CSCs and their FBS tumour pairs (#1247, #1076) was evaluated by immunofluorescence and cytofluorimetric analysis. We checked the presence of these two molecules both cytoplasmic and membrane staining. In figure 21 we can observe that IL-4 was expressed by all the cell lines both in the cytoplasm (4-8 MRFI) and in membrane (3-16). Different IL-4R membrane expression was detectable in 1247 CSC (2 MRFI), 1076 GFD CSC (2 MRFI) and 1076 GFI CSC (2 MRFI) that was absent in FBS lines counterparts, while the cytoplasm expression of the IL-4R was heterogeneously detectable in both CSC and FBS line (2-9 MRFI) and only 1247 FBS was found to lack the expression of this molecule at the cytoplasm level.

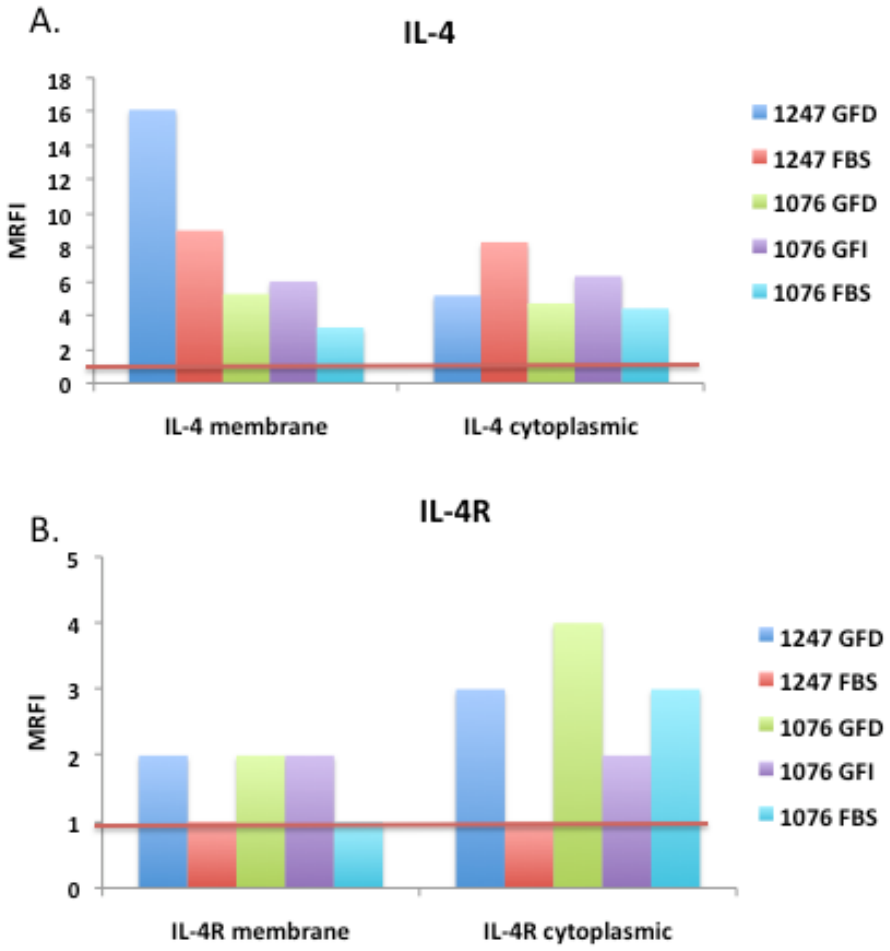


Fig.21 *IL-4 and IL-4R expression by CSC vs. FBS tumor cells.* Expression of IL-4 (panel A) and IL-4R (panel B) by CSC vs. FBS cell lines (#1247, #1076) evaluated by Immunofluorescence (IF) and cytofluorimetric analysis. Data are represented as MRFI (Relative Mean of Flourescence Intensity) that is the ratio between the mean of flourescence intensity of cells stained with the selected mAb and that of the negative control. Significant value were considered ≥ 2 MRFI

The release of some immune-regulatory cytokines: IL-4, IL-5 and IL-13 by CRC CSCs and their FBS tumour pairs (#1247, #1076) was evaluated by EIA, after 48h of the *in vitro* culture, with or without IFN-γ (1000U/ml) (Figure 22).

Surprisingly, we observed that none of the cell lines analyzed, both CSC and FBS, release these cytokines in culture medium. On the contrary, after treatment with IFN-γ for 48h, only one out 5 lines: 1076 CSC GFI produced detectable levels of IL-4 (0.4 pg/ml), IL-5 (2.3 pg/ml) and IL-13 (0.8 pg/ml).

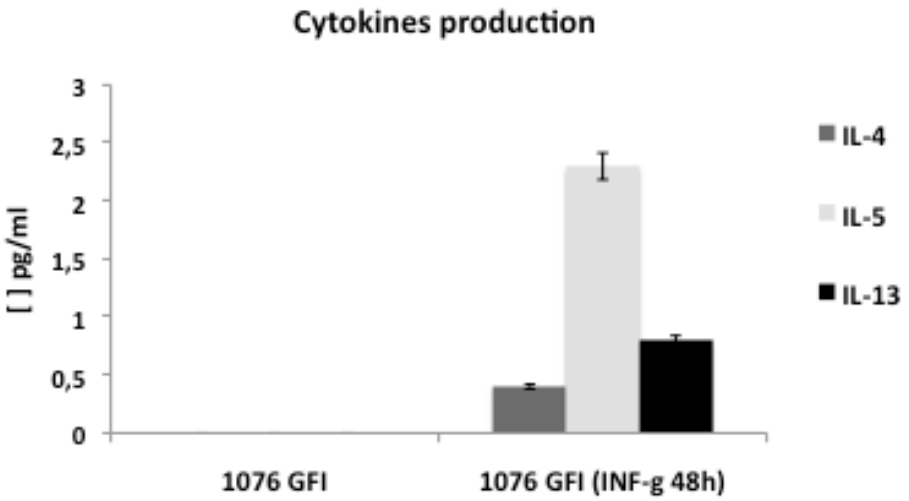


Fig.22 *Detection of IL-4, IL-5 and IL-13 release by 1076 CSC GFI tumour cells, treated or not with the IFN-γ (1000U/ml).* The release of cytokines in the 48h culture supernatants was determined by EIA assay and values were indicated as pg/ml. Results represent averages of triplicates with an SD of ≤10%

6.13 Prostaglandin-E2 (PGE2) release by CRC CSCs vs FBS cells.

Figure 23 reports the results of the secretion, evaluated by EIA, by CSCs vs. FBS tumour cells of Prostaglandin-E2 (PGE2), after 72h of the *in vitro* culture. We observed that levels of PGE2 released by CRC CSC were comparable with that produce by the peripheral blood mononuclear cells (PBMCs), obtained by healthy donors and used as internal control of the experiment. Surprisingly, 1247 FBS and 1076 FBS released high levels of PGE2 (116 and 2086 pg/ml respectively), superior to that of CSCs (29-65pg/ml) and of PBMCs (52 pg/ml) as well.

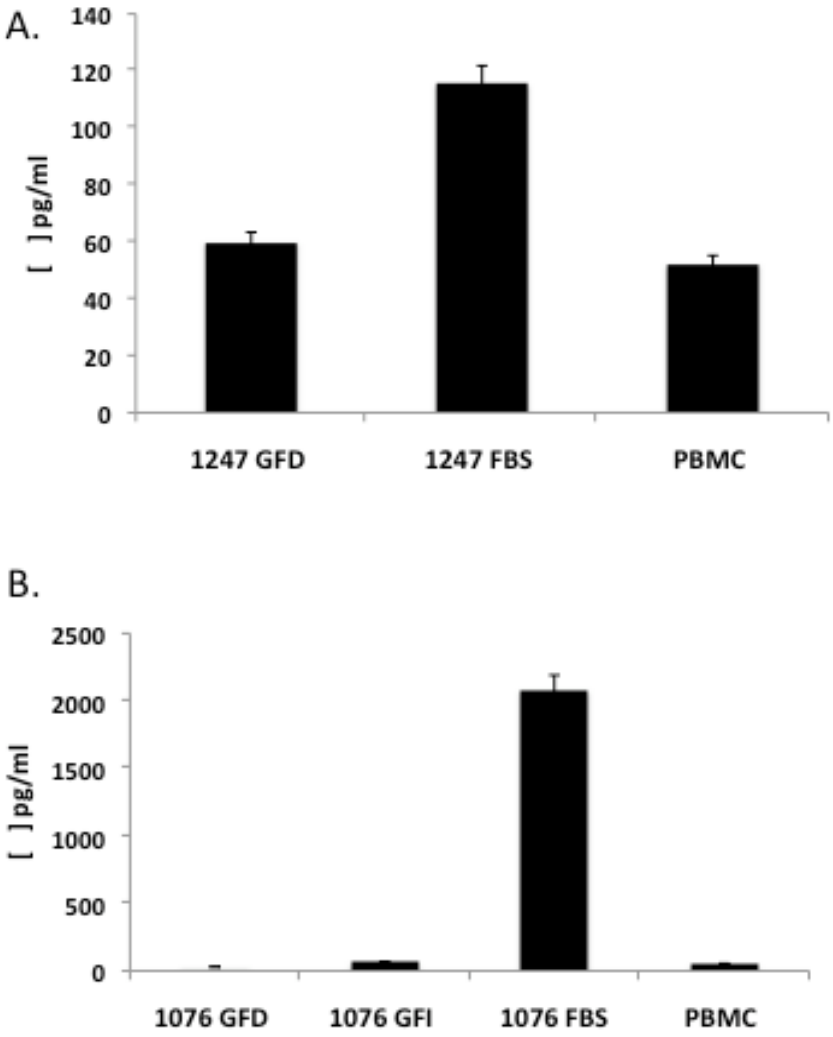


Fig.23 *Detection of PGE-2 release by CSC and FBS tumor cells.* The release of PGE2 in the 72 h culture supernatants by 1247 CRC CSC and FBS tumor cells (Panel A) and 1076 CRC CSC and FBS tumor cells (Panel B). PBMCs were used as internal control. PGE2 levels were determined by EIA assay and values were indicated as pg/ml. Results represent averages of triplicates with an SD of ≤10%

6.14 Isolation *in vitro* of anti-CRC T lymphocytes by stimulation with autologous CSCs or FBS tumor cells.

To assess whether CRC CSCs may represent targets for T cell–mediated immune reactions, PBMCs isolated from two CRC patients (#1076 and #1247) were stimulated *in vitro* with autologous irradiated CSC or FBS tumour cells (mixed lymphocytes tumour cell culture, MLTC), and after 3 to 5 weeks of re-stimulations, the specific reactivity of T lymphocytes was evaluated by cytokine release assays (IFN-γ ELISPOT) (Figure 24).

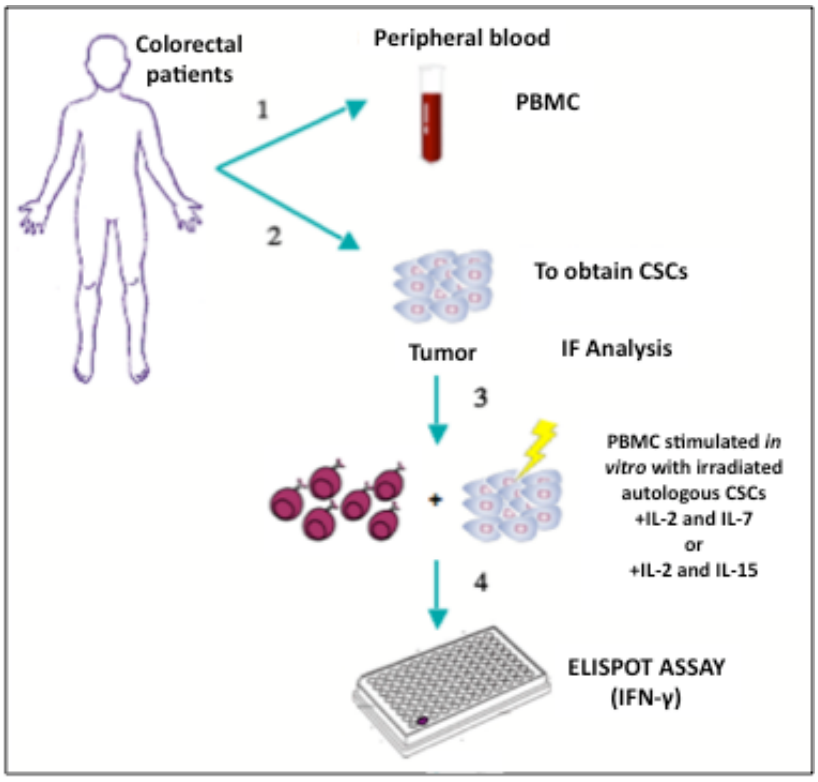


Fig. 24 *Schematic representation of mixed lymphocytes tumor cell culture (MLTC).*

As shown in Figure 25, three independent T-cell lines were isolated *in vitro* following incubation of #1076 lymphocytes with autologous 1076 CSC GFD, 1076 CSC GFI or 1076 FBS cells; their reactivity was measured by the release of IFN-γ, (numbers of spots / 5x 10^3 cells).

T lymphocytes, grown *in vitro* with a combination of IL-2 and IL-15 or IL-21 cytokines and stimulated with irradiated 1076 CSC GFD specifically recognized the autologous GFD CSCs in a MHC class I-restricted manner, as shown by the inhibition of IFN-γ secretion in the presence of W6/32 mAb (82% of reduction of the number of spots) and 1076 FBS (80% of reduction of the number of spots). They also partially recognized 1076 CSC GFD (17% o of reduction of the number of spots) and 1076 FBS (23% of reduction of the number of spots) in a MHC class II-restricted manner (Panel A), as shown by the inhibition of IFN-γ secretion in the presence of L243 mAb. On the contrary T lymphocytes weakly recognized #1076 CSC GFI (59.5 spot, p=0.006) (Figure 25, Panel A). This line was previously found to be weakly immunogenic and also the cytofluorimetric analysis of APM components (Table 1) revealed lower or negative levels of expression of APM molecules than the 1076 FBS counterpart.

Differently, T lymphocytes grown *in vitro* with a combination of IL-2 and IL-21 and stimulated with irradiated autologous 1076 CSC GFI (Figure 25, Panel B), recognized at high levels (202 spot, p=0.004) the 1076 FBS cells both in MHC class I (31% of spots inhibition) and MHC class II (28% of spots inhibition) restricted manner, and less efficiently (22 spot, p=0.16), the 1076 GFD CSC. No reactivity was observed against the 1076 GFI CSC.

At last T lymphocytes, grown *in vitro* with a combination of IL-2 and IL-21

cytokines and stimulated with irradiated 1076 FBS for up to five weeks, specifically recognized autologous 1076 FBS (233 spots, p=0.0007) both in a MHC class I (30% of spots inhibition) and in a MHC class II (31% of spots inhibition) manner (Panel C). Thus 1076 CSC GFD (43 spots, p=0.01) and 1076 CSC GFI (35 spots, p=0.03) are also detected with less efficiently, mainly in a MHC class-I restricted manner (Figure 25, Panel C). T lymphocytes were also stimulated *in vitro* with the mitogen PHA, used as positive control (170-355 spots versus 18-35 spots/$5x10^4$ cells, p=0.032).

In conclusion, these data suggest that T-cell cultures stimulated with 1076 CSC GFD and 1076 FBS recognized autologous tumour cells mainly in a MHC class I-restricted manner and partially in MHC class II-restricted manner; interestingly these cells recognized more efficiently FBS tumor cells compared to CSCs. On the contrary T-cell cultures stimulated with 1076 CSC GFI did not recognized autologous tumour cells but efficiently recognized 1076 FBS (202 spot, p=0.004) and less efficiently, the 1076 GFD CSC .

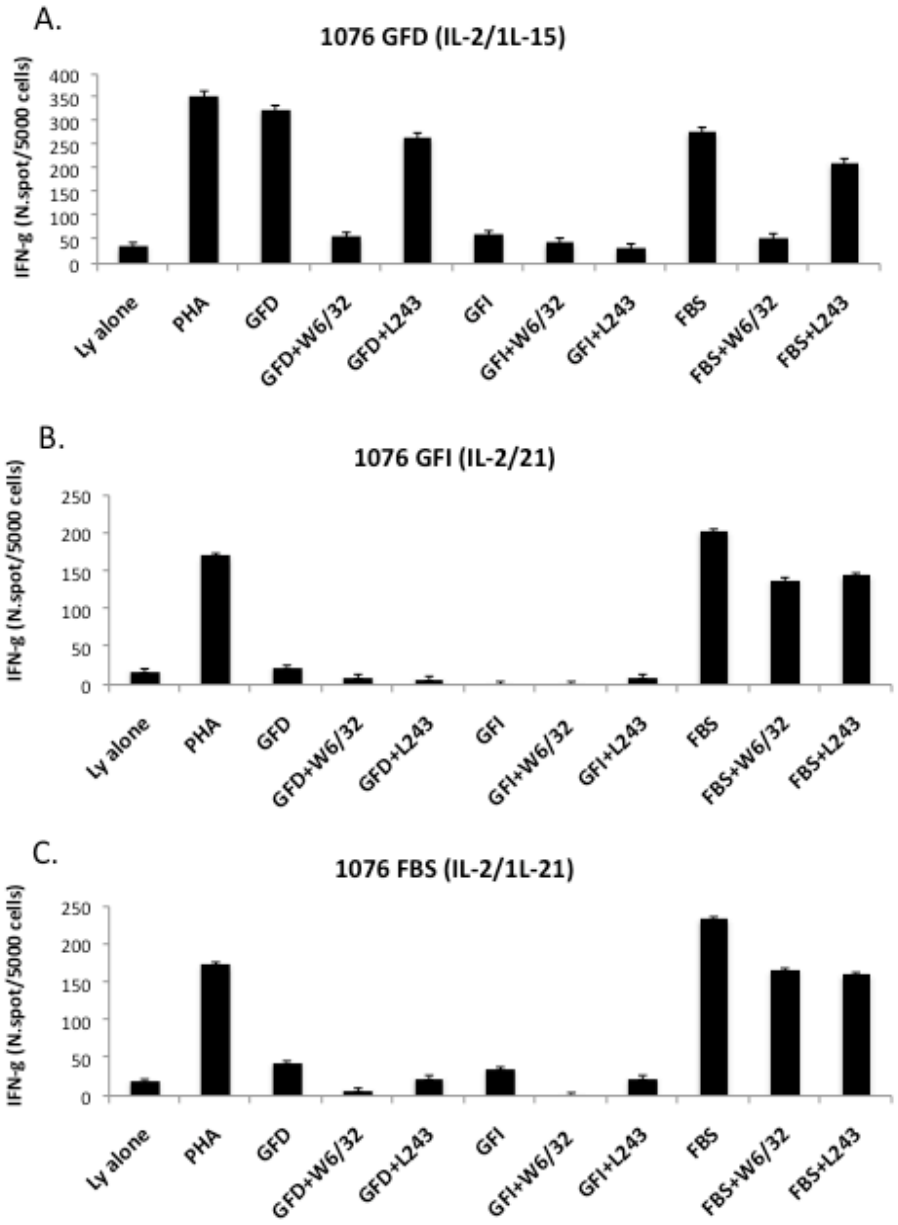


Fig.25 *The reactivity of 1076 T lymphocytes against autologous CRC CSCs or FBS tumour cells.* PBMCs isolated from CRC patient 1076 were stimulated *in vitro* with autologous irradiated 1076 CSC GFD (panel A), 1076 CSC GFI (panel

B) and 1076 FBS (panel C), following 3-4 weeks of culture, the reactivity against autologous CRC CSCs and FBS was assessed by IFN-γ release (ELISPOT assay). The cytokine secretion was determined following stimulation of T cells with autologous CSCs (GFD and GFI) and FBS, autologous CSCs (GFD and GFI) and FBS pretreated with anti-MHC class I (W6/32) or anti-MHC class II (L243) mAbs and, as positive control, T lymphocytes treated with the mitogen PHA. Data are expressed as number of spots per 5×10^3 cells. Results represent averages of triplicates with an SD of ≤10%; statistical analysis of differences between means of IFN-γ released by T cells was done by two-tailed t test.

Similar experiments have been carried out for patient #1247. In this context PBMCs, isolated from this patient and grown *in vitro* in the presence of IL-2 and IL-7, were stimulated with irradiated 1247 CSCs or 1247 FBS: (i) alone, (ii), treated with anti-IL-4 mAbs and/or (iii) pre-treated for 48h with IFN-γ 1000 U/ml.

Figure 26 represents data related to the incubation of T lymphocytes with autologous 1247 CSC alone, 1247 CSC pre-treated for 48h with IFN-γ (1000 U/ml) and 1247 CSC treated with anti-IL-4 mAbs. T lymphocytes isolated by the stimulation *in vitro* with autologous CSCs (Figure 26, Panel A) specifically recognized 1247 CSC (268 spots, p=0.009) but without MHC-restricted manner. Of note, the phenotype analysis of these lymphocytes revealed the presence of 20% NK cells ($CD3^-CD16^*CD56^+$, data not shown). On the contrary, these T lymphocytes recognized the autologous1247 FBS (226 spots, p=0.0002) in the context of MHC class I (23% of reduction of the number of spots in the presence of W6/32 mAb). MHC-independent reactivity against CSC or FBS cells was detectable by T cells stimulated with 1247 CSC pre-treated for 48h with IFN-γ 1000 U/ml (Figure 26, Panel B).

Interestingly, MLTC cultured with anti-IL-4 mAbs specifically recognized autologous 1247 CSC (40% of reduction of the number of spots in the

presence of L243 mAb, Figure 26, Panel C) and 1247 FBS (24% of reduction of the number of spots in the presence of L243 mAb, Figure 26, Panel C) in a MHC class II-restricted manner (Panel B). Similar results were obtained for the pre-treatment of target cells for 48h with IFN-γ (1000 U/ml).

MLTCs from patient #1247 have been generated *in vitro* also by the usage of autologous FBS cells as stimulators (Figure 27).

In Panel A lymphocytes, stimulated with autologous 1247 FBS alone, recognized both the autologous 1247 CSC (115 spots, p=0.002) and 1247 FBS (96 spots, p=0.002) but with no specific MHC-restriction. The phenotype analysis revealed a presence of about 10% of NK cells ($CD16^+$, $CD56^+$ and $CD3^-$). While these lymphocytes could recognized with weak inhibition by L243 mAb the autologous CSC but not the FBS cells (Figure 27, Panel A).

MHC-independent recognition of CSCs pre-treated with IFN-γ or FBS pre-treated with IFN-γ was observed by PBMC stimulated *in vitro* with 1247 FBS pre-treated with IFN-γ (Figure 27, panel B); only a weak inhibition of cytokine release (30% of the reduction of the number of spot) was detected in the recognition of FBS cells incubated with the anti-MHC class II L243 mAb (Figure 27, Panel B).

The incubation of this MLTC with anti-IL-4 mAb did not affect its CSC or FBS recognition except for the induction of MHC class II-restricted (25% of spot reduction) reactivity against FBS cells pre-treated with IFN-γ (Figure 27, panel C).

Taken together, these results indicate that T cell responses could be obtained in one CRC patient (#1076) directed against CSC and FBS cells, though with higher reactivity for FBS tumor cells, while in patient

#1247 NK-mediated responses could be isolated. Nevertheless, in the latest case the blocking of IL-4 could restore T cell-, mainly $CD4^+$ mediated responses.

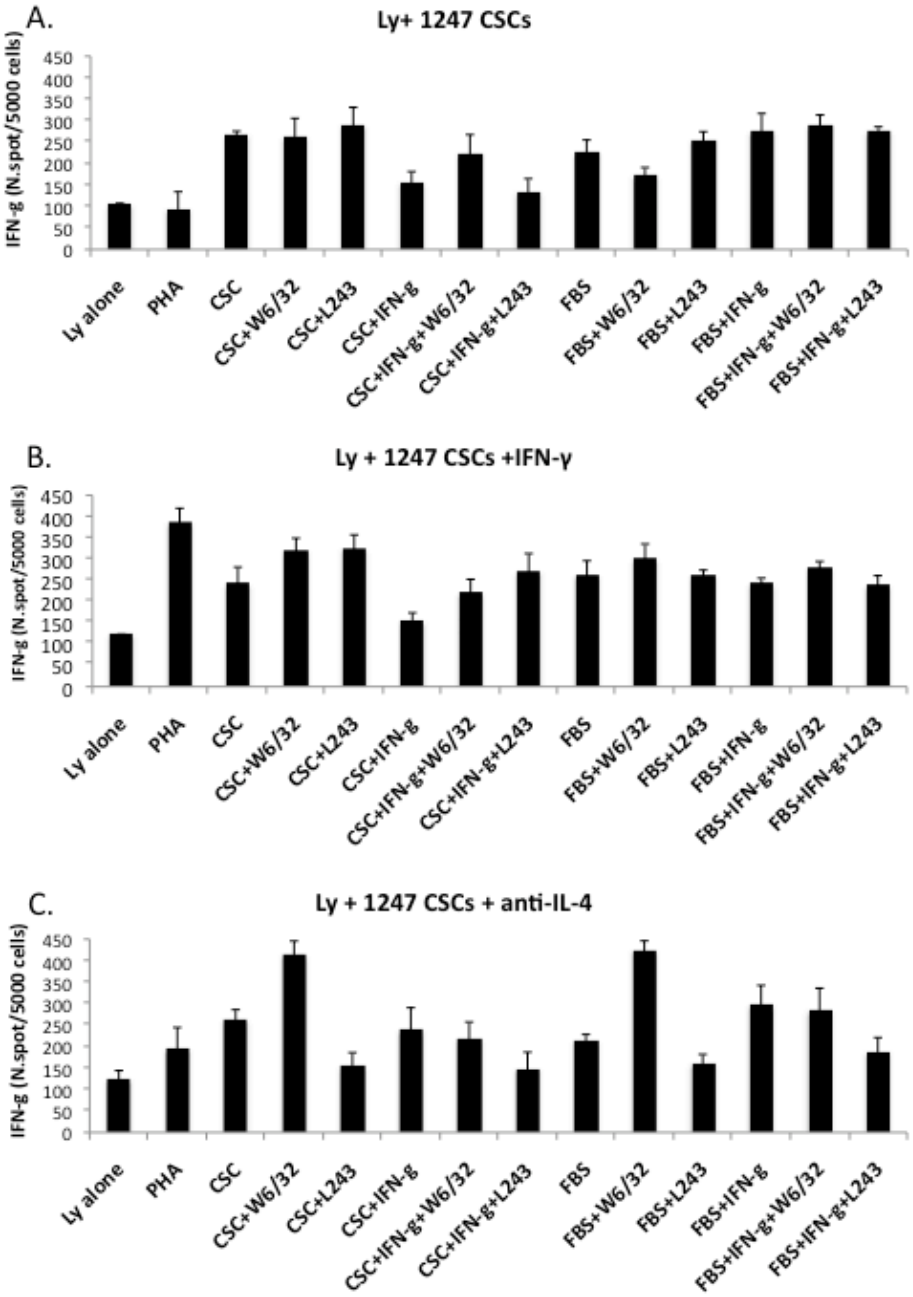
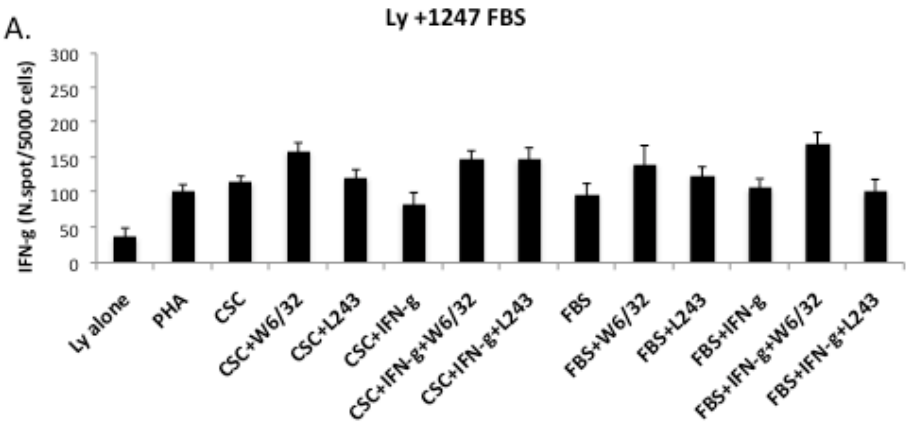

A.
Ly+ 1247 CSCs
IFN-g (N.spot/5000 cells)
450
400
350
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
FBS
FBS+W6/32
FBS+L243
FBS+IFN-g
FBS+IFN-g+W6/32
FBS+IFN-g+L243
B.
Ly + 1247 CSCs +IFN-γ
IFN-g (N.spot/5000 cells)
450
400
350
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
FBS
FBS+W6/32
FBS+L243
FBS+IFN-g
FBS+IFN-g+W6/32
FBS+IFN-g+L243
C.
Ly + 1247 CSCs + anti-IL-4
IFN-g (N.spot/5000 cells)
450
400
350
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
FBS
FBS+W6/32
FBS+L243
FBS+IFN-g
FBS+IFN-g+W6/32
FBS+IFN-g+L243

Fig 26. *Reactivity of 1247 T lymphocytes against autologous CRC CSCs.* PBMCs isolated from CRC patient 1247 were stimulated *in vitro* with autologous irradiated 1247 CSCs (panel A), 1247 CSCs pretreated for 48h with IFN-γ 1000 U/ml (panel B) and1247 CSCs treated with anti-IL-4 mAbs (panel C), following 3 weeks of culture, the reactivity against autologous CRC CSCs and FBS was assessed by IFN-γ release (ELISPOT assay). Cytokine secretion was determined following stimulation of T cells with autologous CSCs and FBS, pretreated or not for 48h with IFN-γ 1000 U/ml, autologous CSCs and FBS, pretreated or not for 48h with IFN-γ 1000 U/ml and pretreated with anti-MHC class I (W6/32) or anti-MHC class II (L243) mAbs, and as positive control, T lymphocytes treated with the mitogen PHA. Data are expressed as number of spot per 5×10^3 cells. Results represent averages of triplicates with an SD of ≤10%; statistical analysis of differences between means of IFN-γ released by T cells was done by two-tailed t test ($p<0.05$).

A.


Ly +1247 FBS
IFN-g (N.spot/5000 cells)
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
FBS
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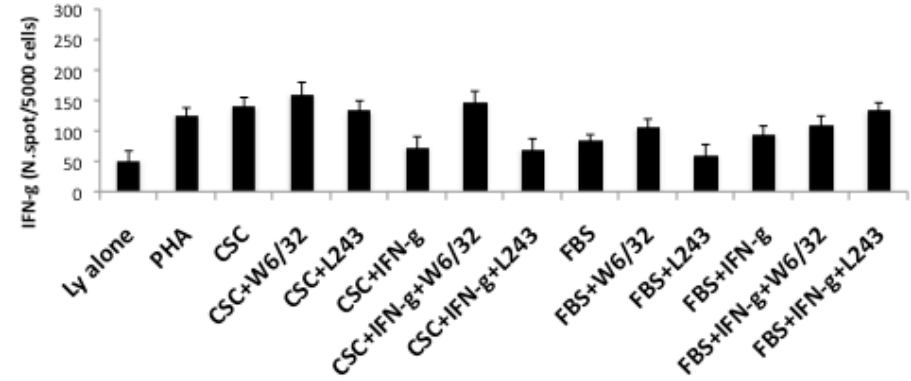
FBS+L243
FBS+IFN-g
FBS+IFN-g+W6/32
FBS+IFN-g+L243

B.


Ly + 1247 FBS + IFN-Y
IFN-g (N.spot/5000 cells)
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
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FBS+IFN-g+W6/32
FBS+IFN-g+L243

C.

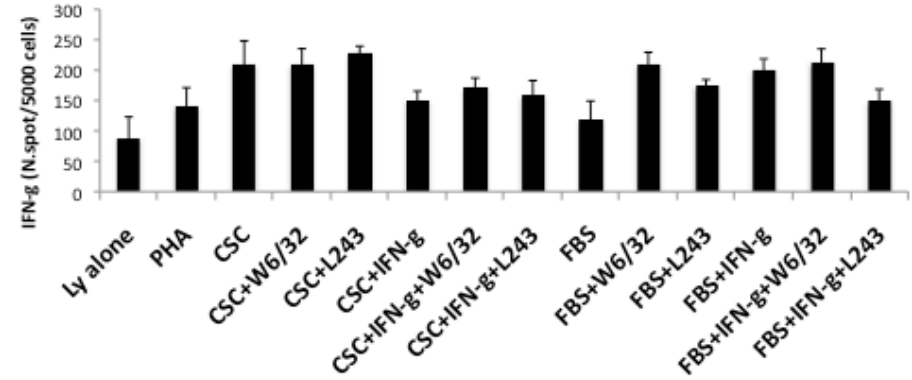
Ly + 1247 FBS + anti-IL-4
IFN-g (N.spot/5000 cells)
300
250
200
150
100
50
0
Ly alone
PHA
CSC
CSC+W6/32
CSC+L243
CSC+IFN-g
CSC+IFN-g+W6/32
CSC+IFN-g+L243
FBS
FBS+W6/32
FBS+L243
FBS+IFN-g
FBS+IFN-g+W6/32
FBS+IFN-g+L243

Fig. 27. Reactivity of 1247 T lymphocytes against autologous 1247 FBS. PBMCs isolated from CRC patient 1247 were stimulated *in vitro* with autologous irradiated 1247 FBS (panel A), 1247 FBS pre-treated for 48h with IFN-γ 1000 U/ml (panel B), and1247 FBS treated with anti-IL-4 mAbs (panel C), following 3 weeks of culture, the reactivity against autologous CRC CSCs and FBS was assessed by IFN-γ release (ELISPOT assay). Cytokine secretion was determined following stimulation of T cells with autologous CSCs and FBS, pretreated or not for 48h with IFN-γ 1000 U/ml, autologous CSCs and FBS, pretreated or not for 48h with IFN-γ 1000 U/ml and pre-treated with anti-MHC class I (W6/32) or anti-MHC class II (L243) mAbs, and as positive control, T lymphocytes treated with the mitogen PHA. Data are expressed as number of spot per 5×10^3 cells. Results represent averages of triplicates with an SD of ≤10%; statistical analysis of differences between means of IFN-γ released by T cells was done by two-tailed t test ($p<0.05$).

7. Discussion

In this work we have carried out for the fist time an immuno-biological characterization of CRC CSCs. Indeed our group was recently successful in isolating *in vitro,* by mechanical dissociation of tumor specimens and exposure of tumor-derived cells to specific mitogens, CRC CSCs and their FBS-cultured differentiated tumor cells. We observed that CRC CSCs retained the fundamental stem-cell criteria of multipotency: when cultured in medium supplemented with 10% FBS (representing a differentiation agent), CSCs lost the ability to grow in suspension in the form of spheres and displayed adherence dependent growth, showing a differentiated morphology similar to the FBS tumor cells. CRC CSCs express, though heterogeneously, most of the markers associated with CSCs and CRC and in same cases CSCs were found to express higher levels of these molecules than the corresponding FBS cell lines (Figure 11).

Furthermore, the tumorigenic potential of CRC CSCs was higher than that of FBS cells as shown by the serial dilutions and serial transplantation in immunodeficient mice. Indeed the xenografts generated by CRC CSCs: (i) showed a more efficient capacity to initiate tumor growth when injected in nude mice, (ii) generated tumour in nude mice at lower cell concentrations than FBS tumor cells and (iii) retained the same tumorigenic potential when were re-injected into secondary mice.

Moreover a preliminary morphological analysis of xenografts generated by CRC CSCs injection showed large areas of necrosis than tumor tissues generated by FBS cells. This phenotype correlated with the higher proliferative potential of CRC CSCs and, moreover, the IHC

analysis of CRC and CSC-associated molecules revealed that CSCs xenografts better matched the phenotype of the original tumor.

We have carried out the immunobiological characterization of CRC CSCs by analyzing the expression of MHC-I and MHC-II molecules, NKG2D ligands (MICA/B, ULBP-1-4), APM, immune-regulatory molecules (CTLA-4 and their ligands B7-1 and B7-2, PD-1 and its ligands PDL1 and PDL-2, B7-H2 and B7-H3).

Both CRC CSC and FBS lines were found weakly positive for MHC-I and negative for MHC-II, while NKG2D ligands were commonly detected in both cells types (Figure 17). The APM was more defective in CRC CSC than in FBS cell lines, in particular this was observed for patient #1247 (1247 GFD CSC line) and for patient # 1076 (1076 GFI CSC line) (Table 2). All CSCs and FBS cell lines were homogeneously positive for CTL-4, PD-1 and, although at higher levels, for PD-L1 and B7-H3 (Figure 19). These molecules act as negative modulator of T-cells response because they are able to inhibit the 'second signal' required for optimal T cell activation (Macian *et al.*, 2004). Studies of PD-L1 expression in tumors show that this molecule may facilitate advancement of tumor stage and invasion into deeper tissue structures and its expression strongly correlates with bad prognosis (Keir *et al*., 2008). In addition B7-H3 has been described to correlate with a more advanced tumor grade in CRC (Sun J. *et al.,* 2010) and represent a new promising target for immunotherapy even if its role in T-cell responses and especially in tumor immunity has yet to be elucidated.

Thus, low level of expression of APM components, the lack of costimulatory molecule ligands, such as B7-1 and B7-2 and the expression of the immune-regulatory molecules, such as CTLA-4, PD-1,

PD-L1 and B7-H3 may render these cells weakly immunogenic.
The cytokine IL-4 was expressed by all the cell lines both in the cytoplasm and both surprising in membrane while IL-4R was mainly was weakly detectable on the membrane of CSCs and was absent in FBS lines counterparts (Figure 21).

These data confirm the possibility of an autocrine production by CRC CSC and also FBS line of IL-4, that may protect themselves from apoptosis as recently described (Todaro M *et al,* 2007).
Unexpectedly the Prostaglandin-E2, know to contribute to cellular immune suppression in cancer patients, including CRC (Ahmadi *et al.,* 2008), was released at high level by FBS lines but not by CSCs. We aim to investigate this mechanism in further studies.
In addition, T lymphocytes isolated from the peripheral blood of CRC patient #1076, stimulated *in vitro* with autologous CSCs or FBS cells efficiently recognized of FBS cell lines (in a MHC class I-restricted manner and partially in MHC class II-restricted manner) while showing weak reactivity against 1076 GFD CSC and 1076 GFI CSC (Figure 25). Indeed 1076 GFI CSC line was found to express lower levels of APM molecules than the 1076 FBS counterpart and it was found to produce detectable levels of pro-inflammatory cytokines: IL-4, IL-5 and IL-13, manly associated with a TH2 immune-response.
The MLTCs isolated from the CRC patient #1247 showed MHC-independent recognition of both 1247 CSCs and 1247 FBS cells suggesting that NK-type reactivity occurred in this patient (Figure 26-27). Indeed, the phenotype analysis confirmed that 20-30% of NK cells were present in these cultures (data not shown). Notably, when MLTC were cultured *in vitro* with the anti-IL-4 mAb MHC class II-restricted recognition

of autologous CSC occurred, indicating that the blocking of IL-4 could rescue CD4+ T cell responses. Though the mechanism is not elucidated yet, this evidence suggests that by blocking the autocrine signal of IL-4 associated with CRC CSC, efficient T cell immune responses can be obtained. This issue will be addressed in further studies.

The expression of a panel of TAAs, analysed by IF and confocal microscopy analysis, revealed that both CSCs and FBS tumor cell lines homogenously expressed high levels of CRC associated antigen COA-1 able to induce both $CD8^+$ and $CD4^+$ T-cell-mediated responses in CRC patients (Maccalli C. et al., 2008), and of Survivin, an inhibitor of apoptosis, whose over-expression was recently found to correlate with increased invasion and metastasis of colorectal cancer (Xiao-Yuann Chu et al., 2011), suggesting that these antigens can represent candidate target molecule for CSC-directed T cell responses as well.

In conclusion, we have isolated from CRC primary human tumors CSCs with the following properties: (i) to grow in suspension in the form of spheres, (ii) to express markers associated with CRC and CSCs, (iii) to show multipotency properties and (iv) to retain an high tumorigenic potential when injected in immunodeficient mice.

Our data indicate a lower immunogenic profile of CSCs compared to FBS counterparts and the expression by these cells of immune-regulatory molecules (CTLA-4, PD-1, PD-L1 and B7-H3). Thus the lack of costimulatory molecule ligands, such as B7-1 and B7-2 and the down-modulation of MHC and APM molecules may render these cells weakly immunogenic. These results have been confirmed by the functional analysis of MLTC experiments, indicating that, in some cases, CSC can be more susceptible to NK- than T-cell mediated recognition and that the

blocking of IL-4 signaling can rescue $CD4^+$ T cell reactivity against CSCs. Our findings may be useful for the design of immunotherapy protocols for CRC patients and indicate the need to identify new immunomodulatory agents that can efficiently rescue the immunogenicity of CSCs in order to be targeted by T cell-mediated immune response.

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