UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato
MEDICINA MOLECOLARE

Curriculum
MEDICINA MOLECOLARE DELLE REAZIONI IMMUNI ED INFIAMMATORIE
(CICLO XXVI)

GENERATION OF TUMOR-SPECIFIC CYTOTOXIC T-LYMPHOCYTES FROM PERIPHERAL BLOOD OF COLORECTAL CANCER PATIENTS FOR ADOPTIVE T-CELL TRANSFER

Settore disciplinare: MED/07

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Anno Accademico 2012/2013

Ad Alessandro,

affinché i progressi della ricerca

possano, un giorno, impedire

ai piccoli angeli di volare via

ABSTRACT

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death in the developed Western countries.
Adoptive T-cell transfer (ACT) refers to an immunotherapeutic approach in which anti-tumor T lymphocytes, usually the tumor infiltrating lymphocytes (TIL), are identified, grown ex vivo and then re-infused into the cancer patient.
ACT of EBV-specific T-cell lines and T Cytotoxic Lymphocytes (CTLs) for the therapy of EBV-induced lymphomas is the best demonstration of clinically efficacious ACT, but there are many evidences also for leukemia and multiple myeloma.
As regards to the solid tumors, ACT using autologous TIL, grown ex-vivo and then re-infused into the cancer patient, has emerged as an effective treatment for metastatic melanoma and renal cell carcinoma (RCC), that are the most immunogenic tumors in humans. Randomized clinical trials are ongoing for gastric cancer, hepatocellular carcinoma and lung cancer.
These approaches mainly use the TIL and the definition of tumor associated antigen (TAA), tumor specific antigen (TSA) or cancer testis antigen (CTA), that are generally correlated with tumor progression and immunogenicity in various types of cancer.
However these antigens are often found to be poorly expressed in CRC, and few is known about their relationship with this type of neoplasia. In addition, although a clear association between TIL and clinical outcome of CRC has been documented, active and adoptive immunotherapy do not play yet an important role in the treatment of advanced CRC.
In order to develop an ACT protocol for CRC treatment, we designed an experimental approach that does not require neither the definition of molecular defined tumor antigens, nor the availability of TIL. Our strategy was based on the in vitro stimulation of patient's $CD8^+$-enriched T-cells from peripheral blood mononuclear cells (PBMCs) with dendritic cells (DCs), pulsed with apoptotic tumor cells as a source of tumor antigens, in order to generate autologous CTLs with strong anti-tumor activity.
In this study, 78 CRC patients were enrolled. Tumor biopsies were obtained at surgery, together with 100 ml of heparinized peripheral blood (PB). Tumors were mechanically dissociated to a single-cell suspension and cultured to obtain tumor cell line from each patient. DCs were generated from previously separated PBMCs, using a magnetic positive selection of $CD14^+$ monocytes, cultured in presence of recombinant human Interleukin-4 (rh IL-4) and recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (rh GM-CSF). Anti-tumor CTLs were elicited in co/micro-culture using DCs as antigen-presenting cells, autologous apoptotic tumor cells as source of antigens and T $CD8^+$ lymphocytes enriched effectors, with weekly stimulation. CTLs Interferon-γ (IFN-γ) secretion was assessed by ELISpot assay to evaluate their activation in response to autologous tumor.
Tumor cell lines were obtained from 20 out of 78 patients (25,6%), because gut intestinal flora had adversly affected the establishment of primary tumor cell line

and a loss of expansion of tumor cells was observed. DCs were generated from 26 patients, but only 6 patients had the corresponding tumor cell line, indispensable for the co-culture setting up. This was the reason why co/micro-cultures were set up only for 6 patients. ELISpot assay was performed at the end of co/micro-culture stimulations to evaluate effectors IFN-γ secretion. ELISpot results showed that strong and significant IFN-γ secretion was detected at the third, fourth and fifth stimulations for one patient and at the second for another patient, whereas for three patients a weak secretion was detected during the second and third stimulations.

Although our immunological study must be performed on an increased number of CRC patients, and the CTLs expansion, together with CTLs lytic ability against autologous tumor cells, must be still performed, our results suggested that the generation of tumor-specific CTLs could be useful for supporting an ACT approach in CRC.

RIASSUNTO

Il carcinoma del colon retto (CRC) è il terzo tipo di tumore più frequente al mondo e la quarta causa di morte per tumore nei paesi Occidentali.
La terapia cellulare adottiva (o trasferimento adottivo di cellule T) (ACT) è un approccio immunoterapeutico in cui, i linfociti T con attività anti-tumorale, generalmente i linfociti T infiltranti il tumore (TIL), vengono identificati, espansi ex vivo e reinfusi nel paziente neoplastico.
La migliore dimostrazione di efficacia clinica della ACT è stata ottenuta nei linfomi associati al virus di Epstein Barr (EBV), utilizzando linee di cellule T-EBV specifiche e linfociti T citotossici (CTLs), ma buoni risultati sono stati ottenuti anche per diversi tipi di leucemie e per il mieloma multiplo.
Per quanto riguarda i tumori solidi, la ACT, utilizzando TIL espansi ex vivo e poi reinfusi nel paziente, si è rivelata un trattamento terapeutico efficace per pazienti affetti da due tumori particolarmente immunogenici quali il melanoma e il carcinoma a cellule renali (RCC). Studi clinici di ACT sono attualmente in fase di sperimentazione per il carcinoma gastrico, il carcinoma epatocellulare e il cancro del polmone.
Questi approcci sfruttano principalmente l'utilizzo di TIL e l'identificazione di antigeni tumore associati (TAA), antigeni tumore specifici (TSA) o degli antigeni del cancro testicolare (CTA), dal momento che essi sono generalmente correlati con la progressione neoplastica e con l'immunogenicità di diversi tipi di tumore.
Tuttavia, questi antigeni sono spesso poco espressi nel CRC e le loro interazioni con questo tipo di tumore sono ancora poco definite.
In aggiunta, sebbene sia stata riportata una chiara associazione tra TIL e "outcome" clinico nei pazienti affetti da CRC, sia gli approcci di immunoterapia attiva che adottiva non giocano ancora un ruolo chiave nel trattamento del CRC in fase avanzata.
Per sviluppare un protocollo di ACT nel CRC, abbiamo disegnato un approccio sperimentale che non necessita nè dell'identificazione di antigeni tumorali molecolarmente definiti, nè dell'isolamento dei TIL. La nostra strategia prevede la stimolazione dei linfociti T $CD8^+$ del paziente, ottenuti a partire dall'isolamento delle cellule mononucleate del sangue periferico (PBMCs), con le cellule dendritiche (DCs) dello stesso paziente, pulsate con le cellule tumorali autologhe condotte in apoptosi per avere una fonte eterogenea di antigeni tumorali, al fine di generare ex-vivo CTLs con una forte attività antitumorale.
Nel nostro studio sono stati arruolati 78 pazienti affetti da CRC. Le biopsie tumorali sono state prelevate in sede operatoria, contemporaneamente ad un prelievo di sangue periferico di circa 100 mL.
Il tumore è stato dissociato meccanicamente fino ad ottenere una sospensione cellulare, che è stata poi messa in coltura per l'ottenimento di linee cellulari primarie tumorali da ciascun paziente. Le DCs sono state prodotte a partire dalla differenziazione dei monociti, isolati magneticamente con beads $CD14^+$ dai PBMCs, coltivati in presenza di Interleuchina 4 ricombinante umana (rh IL-4) e del Fattore stimolante le colonie granulocitarie-macrofagiche ricombinante umano (rh GM-CSF).

Si è quindi cercato di favorire l'attivazione dei CTLs autologhi contro il tumore co-coltivandoli con le DCs, le cellule presentanti l'antigene per eccellenza, caricate con cellule tumorali apoptotiche, che costituiscono la nostra fonte eterogenea di antigeni tumorali, attraverso stimolazioni settimanali ed in presenza di opportuni mediatori. Per valutare la secrezione di Interferon-γ (IFN-γ) da parte dei CTLs, e quindi la loro attivazione in risposta al tumore, è stato utilizzato il saggio immunoenzimatico ELISpot.

Le linee cellulari tumorali sono state ottenute da 20 pazienti su 78 arruolati, poichè la flora intestinale da un lato, e una mancata espansione cellulare dall'altro, ne hanno compromesso l'ottenimento. Le DCs sono state generate da 26 pazienti, ma soltanto per 6 pazienti è stata ottenuta la corrispondente linea cellulare tumorale con cui poter allestire la co-coltura. Questo è il motivo per cui sono state allestite solo 6 co-colture e il test ELISpot è stato quindi effettuato per valutare la secrezione di IFN- γ a partire dalla fine delle stimolazioni settimanali degli effettori di questi 6 pazienti. I risultati del saggio ELISpot hanno mostrato una forte e significativa secrezione di IFN-γ alla terza, alla quarta e alla quinta stimolazione per un paziente, e alla seconda per un altro paziente, mentre in 3 pazienti una debole secrezione della citochina è stata riscontrata durante la seconda e la terza stimolazione.

Sebbene il nostro studio immunologico debba essere condotto su una più ampia casistica di pazienti affetti da CRC, e l'espansione dei CTLs, così come la loro capacità litica nei confronti delle cellule tumorali autologhe debba essere ancora messa a punto, i nostri risultati ci suggeriscono che l'ottenimento di CTLs tumore-specifici potrebbe essere molto utile per supportare approcci di ACT nel CRC.

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LIST OF SYMBOLS

5-FU	5-Fluorouracile
7-AAD	7-aminoactinomycin D
ACT	Adoptive Cellular Therapy
AFP	Alpha Fetoprotein
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
APC	Allophycocyanin
APCs	Antigen Presenting Cells
BCG	Bacillus Calmette-Guerìn
Bcl-2	B cell lymphoma-2
Bcl-xL	B cell lymphoma-extra Large
CAIX	Carbonic Anhydrase IX
CEA	Carcino-Embryonic Antigen
CFU	colony-forming units
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer
CTA	Cancer Testis Antigen
CTLA-4	Cytotoxic T-Lymphocytes antigen four
CTLs	Cytotoxic T-Lymphocytes
CycB	Cyclophilin B
DCs	Dendritic Cells
DMSO	Dimethyl Sulfoxide
EBNA	Immunodominant Epstein-Barr Virus Nuclear Antigen
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunoabsorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
EpCAM	Epithelial Cell Adhesion Molecule
FAP	Familial Adenomatous Polyposis
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GCC	Guanylyl cyclase C
rh GM-CSF	Recombinant human Granulocyte-Macrophage Colony Stimulating Factor
GMP	Good Manufacturing Practices

gp100	Glycoprotein 100
GUCY2C	Guanylate cyclase 2C
Her2/neu	Human epithelial growth factor receptor-two
HLA	Human Leukocyte Antigen
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HSC	Hematopoietic Stem Cell
HSP	Heat Shock Proteins
iDCs	Immature Dendritic Cells
IBD	Inflammatory Bowel Disease
ICS	Intracellular Staining
IDO	Indolamine 2,3-Dioxigenase
IFN-γ	Interferon gamma
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-15	Interleukin-15
rh IL-2	Recombinant human Interleukin-2
rh IL-4	Recombinant human Interleukin-4
rh IL-7	Recombinant human Interleukin-7
rh IL-12	Recombinant human Interleukin-12
JPS	Juvenile Polyposis Syndrome
LAK	Lymphokine activated killer cells
LMP-1	Epstein-Barr virus Latent Membrane Protein-one
LPS	Lipopolisaccaride
mAbs	Monoclonal Antibodies
mDCs	Mature Dendritic Cells
MAGE	Melanoma-associated Antigen
MAGE A1	Melanoma-associated Antigen A one
MAP	Myh-Associated Polyposis
MART1	Melanoma Antigen Recognized by T cells one
MC	McConkey
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibitory Concentration
MSA	Mannitol Salt Agar
MSI	MicroSatellite Instability
MUC1	Mucin one
NK	Natural Killer
PAMP	Pathogen Associated Molecular Pattern
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Program Death receptor one
PD-L1	Program Death Ligand one

PE	Phycoerythrin
PE-Cy5	PE-Cyanine5
PerCp	Peridinin Chlorophyll Protein Complex
PI	Propidium Ioduro
PJS	Peutz-Jeghers Syndrome
PS	Phosphatidylserine
PSA	Prostate Specific Antigen
PTLD	Post-Transplant Lymphoproliferative Disease
RBC	Red Blood Cells
RCC	Renal Cell Carcinoma
RNF43	Ring Finger Protein 43
SAP	Sporadic Adenomatous Polyposis
SART3	Squamous cell carcinoma Antigen Recognized by T-cell 3
ScFv	Single Chain variable Fragments
SEREX	Serological analysis of recombinant cDNA Expression library
siRNA	Small interference RNA
SSC	Side Scatter
TAA	Tumor Associated Antigen
TCR	T-cell receptor
TGF-β	Transforming Growth Factor beta
TGFβRII	Transforming Growth Factor Beta Receptor type II
Th cells	T helper cells
TIL	Tumor Infiltrating Lymphocytes
TLR	Toll-Like Receptor
TLR9	Toll Like Receptor 9
TNF-α	Tumor Necrosis Factor alpha
TNM	Tumor Node Metastasis
TOMM34	Translocase of outer mitochondrial membrane 34
T-reg	Regulatory T-cells
TRP-1	Tyrosine-Related Protein-one
TSA	Tumor Specific Antigen
TSA	Tryptic Soy Agar
UV-B	Ultraviolet B
VEGF	Vascular Endothelial Growth Factor

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1. INTRODUCTION

1.1 Colorectal cancer

1.1.1 Epidemiological features

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide (Figure 1). It affects the bowel and the rectum and is rare in people under 40, with almost 85% of cases being diagnosed in persons over 65 years of age [Ferlay et al., 2008, WHO, 2008]. The latest data collected in the United States from the American Cancer Society's show about 103,170 new cases of CRC for 2012 [Chen at al., 2012]. CRC incidence is the same for man and women, while it is the third common type of cancer in men, after the prostate and lung cancer, and the second most common cancer in women after breast cancer [Ferlay et al., 2008, WHO 2008]. Even if CRC mortality has decreased over the last 20 years, it remains the third cause of cancer related mortality, accounting for approximately 600,000 deaths in 2008 worldwide [Antonic et al., 2013].

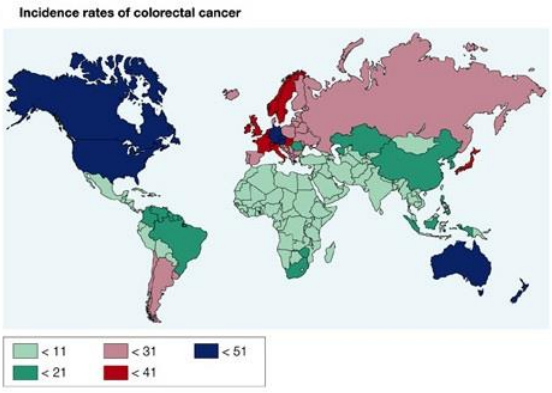


Fig. 1: Prevalence of CRC around the word per 100,000 people [Bingham et al., 2004]. Countries with a high incidence of colon cancer are indicated with blue (North America, Australia); countries with moderate levels in pink or red; and countries with low incidence in green (Asia, Africa).

1.1.2 Colorectal cancer classification, risk factors and genetic profile

Researchers have found several risk factors that may increase a person's chance of developing colorectal polyps or CRC. However, even if a person

with CRC has a risk factor, it is often very hard to know how much that risk factor may have contributed to the cancer.

Age, personal history, family history, racial and ethnic background are the most important components identified. In addition, life style and diet related factors also contribute to the development of CRC as well [Slattery et al., 2000]. CRC can be divided in three subtypes of cancer, depending on whether it is:

a) sporadic, that constitutes the large amount of all CRC (about 80%);

b) inheredited, due to genetic instability;

c) inflammatory, when the development of tumor is successive to the presence of chronic inflammations of gastrointestinal tract, which includes ulcerative colitis and Crohn's disease.

CRC is a paradigm for multistep carcinogenesis with morphological–genomic associations in the adenoma–adenocarcinoma sequence, as introduced by Fearon and Vogelstein in 1990 [Feareon et al., 1990, Muto er al., 1975]. The body of evidence indicates that: 1) some alterations segregate together or inversely as part of a "genetic pathway" [Ilyas et al., 1999]; and 2) functional pathways can be disrupted at different points so that different alterations may have functional "equivalence" in the same pathway [Parsons et al., 2005].

The more frequent genetic alteration that could lead to sporadic CRC is the somatic mutation in *adenomatous polyposis coli (APC)* gene that could be detected in the 80-85% of the patients with nonhereditary sporadic adenomatous polyposis (SAP). Mutation of the *APC* gene is thought to be an early step in the development of CRC [Saif et al., 2010]. This mutational event contributes to the activation of Wnt/βcatenin pathway that actives the transcription of genes such as *c-myc* and *cyclin D*, involved in cell cycle progression.

The *k-ras* gene is one of the oncogenes and it is assumed that the mutation occurs after the *APC* gene in the CRC [Takahashi et al., 2004]; mutation in Ras protein usually cause constitutive activation of Ras GTPase, which leads to over-activation of downstream signaling pathways, resulting in cell transformation and tumorigenesis [Boguski et al.,1993, Rowinsky et al. 1999, Bos et al., 1987, Forrester et al., 1987]; indeed it was demonstrated that activating mutations of *k-ras*, through the subsequent activation of at least 9 pathways [Karnoub et al., 2009], promote cell proliferation, transformation and differentiation [Valencia et al., 1991]. It was hypothesized that this genetic alteration occurs during the early stage of CRC carcinogenesis because the same point mutation was found in the same patient's adenoma and adenocarcinoma, and seems to be related to the increasing size of the tumor. Previously studies have shown that, in clinical specimens, approximately 30% to 50% of CRC harbored *k-ras* mutations [Bos et al., 1987, Forrester et al., 1987, Andreyev et al., 1998, Fox et al., 1998].

The accumulation of next genetic changes like the loss of *p53* tumor suppressor gene could cause the development of invasive CRC [Feareon et al., 1990]. This mutation is the most important point that determines the borderline between the adenoma and the adenocarcinoma [Takahashi et al., 2004] and it is detected in about 75% of CRC patients [Grady et al., 2002].

A minor group of CRC patients (8-15%) is associated with hereditable tendency. This group can be divided in two subgroups: the first develop familial adenomatous polyposis (FAP), due to a germ line mutation of the *APC* gene, and the lifetime incidence of CRC is almost 100% [Burn et al., 2012]; the second includes the patients with Hereditary Non-polyposis Colorectal Cancer (HNPCC or Lynch Syndromes), in which the genetic

anomalies are less defined, but they seem to be associated with mutations of several genes involved in the complex mechanism of DNA mismatch repair [Saif 2010]. However other rare syndromes, such as MYH-associated polyposis (MAP), Peutz-Jeghers syndrome (PSJ), and juvenile polyposis syndrome (JPS) can also increase CRC risk [Centelles, 2012].

1.1.2.1 Infectious agents as risk factors

The long list of cancer risk factors continues to evolve, and in the past few decades has expanded to include infectious agents.

It has been demonstrated the role of small DNA viruses [polyomaviruses, papillomaviruses, and Epstein-Barr virus (EBV)] in the development of various types of cancers such as Merkel cell carcinoma, cervical cancer, Burkitt's lymphoma, Hodgkin's lymphoma, as well the association between *Helicobater pylori* and gastric cancer.

At now, although several works reported that viruses and bacteria can support CRC through direct mutagenesis, secretion of mutagenic products and/or prolonged infection and inflammation that leads to increase epithelial cell proliferation, they are still not recognize as risk factors.

However, Tjalsma and colleagues proposed a model, called "driver-passenger" based on next generation sequencing, in which each stage of CRC has a specific pathogen(s) associated with it [Tjalsma et al., 2012], so there is a observed anatomic predisposition for development of CRC, which can be correlated with the presence and the quantity of bacteria in different part of gastrointestinal tracts (colon and small intestine).

1.1.3 Colorectal cancer staging

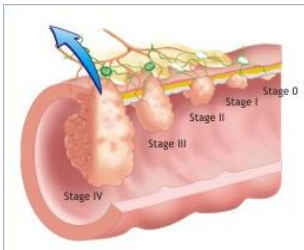
The most common used staging system for CRC is that of the American Joint Committee on Cancer (AJCC), known also as the tumor node metastasis (TNM) system. Two older staging systems include Duke's

[Dukes et al., 1932] and Astler-Coller [Astler et al., 1954] but these are very rarely used today.

The meaning of the three letters combined in AJCC system is:

- T →Describes how far the primary tumor has grown in to the wall of the intestine and if it has grown also in neighboring areas;
- N →Detects the extent of spread to nearby lymph nodes;
- M →Indicates whether the cancer has metastasized to other organs of the body (The more frequent sites of spread in CRC are liver and lungs).

The information from the T, N, M is combined to determine the cancer stage grouping from Stage I (the least advanced), to Stage IV (the most advanced) (Figure 2).



AJCC/TNM Staging		Tumor
Stage 0	Tis (carcinoma in-situ)	Superficially involves the mucosa. Has not grown beyond the mucosa
Stage I	T1N0 T2N0	Invades through mucosa Invades through submucosa
Stage II	T3N0 T4N0	Invades through muscle layers Invades nearby tissues or organs
Stage III	Any T, N1-N3	Lymph nodes involved
Stage IV	Any T, Any N, M1	Distant Spread

Fig. 2: CRC staging
http://alamocitycancercouncil.org/uploads/ACCC_ColonBrochure_Spread.pdf

The Stage 0 is very early stage of CRC, where polyps are formed in the mucosal lining of the colon. In the Stage I polyp develops into a tumor and invades the inner-lining of the mucosa. The Stage II (from A to C) exhibits when the cancer has spread beyond colon but not to the lymph nodes through metastasis. The Stage III (from A to C) shows a spread of all the wall of the colon and of the surrounding lymph nodes. At the Stage IV

cancer has speeded to the other organs, like liver, ovary, testis, intestines [Mishra et al., 2013]

The grade of cancer is another factor used to predict the survival of CRC patients [Ried et al., 1996]. Grade is a description of how closely the cancer looks like normal colorectal tissue when seen under a microscope, and it is indicated from G1 to G4 (where the cancer looks very abnormal). It's deducible that high grade cancers tend to grow and to generate metastasis more quickly than low grade cancers.

1.1.4 Methods of diagnosis

The survival rate changes within the different Stage of CRC, starting from the 95% of the Stage I, to the only 3% of the Stage IV.

Therefore, one of the main objectives is to improve the diagnostic methods for CRC, in order to identify the tumors in the early stage of development.

Methods such as fecal occult blood test, sigmoidoscopy, colonoscopy, virtual colonoscopy and double contrast barium enema offered improvements in the detection rates of CRCs [Strul et al., 2007]. The main limitations of these are: the expensive costs, risks, lack of sensibility in the starting phase of tumor and inconvenience to the patients [National Cancer Institute Factsheets]. So, identifying the early disease biomarkers and setting non-invasive tests, using for example blood or urine, remains a research focus. Tumor biomarkers will offer an opportunity to translate unique CRC biological features into diagnostically pertinent information and would enable personalized treatments.

1.1.5 Conventional treatments

The conventional approaches to treating CRC include: polypectomy and surgery, radiation therapy, chemotherapy and targeted therapy [Mishra et al., 2013].

Polypectomy consists in the surgical removal of the polyps during colonscopy. The surgical removal of pre-cancerous/cancerous lesions has the potential for full recovery of the patient and can be successful for a small and localized tumor outgrown. In addition, surgery is the only way to contrast the chemotherapy and radiotherapy resistant tumors.

Radiotherapy is the careful use of high energy X-rays to control the proliferation of malignant cells. This treatment is directed to the tumor area and to any neighbouring tissues felt to be at risk of containing tumor cells, providing a much larger absorbed dose at the cancer site than in the normal tissue. Depending on the location and the stage of CRC, radiotherapy is recommended in combination with chemotherapy, before or after the surgery. Radiation given before surgery can help shrink tumor, while after keep the cancer from coming back. Possible side effects of radiation include frequent bowel movements, abdominal pain and cramping, rectal discomfort, burning with urination, frequent urination, skin irritation and fatigue, but these symptoms in the majority of cases stop at the end of therapy.

The treatment of metastatic cancers mainly relies on chemotherapy, the method or process of administering a pharmaceutical compound to kiss tumor cells by direct cytotoxicity leading tumor regression. These drugs include alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, enzymes and hormones; these molecules are able to interfere with cell division pathways including DNA replication and chromosomal separation, and unfortunately are not specific for cancer cells. Nowadays the conventional chemotherapy treatment is based on a combination of different drugs, in order to increase the disease free survival. The CRC treatments more commonly used and characterized by the administration of a drug plus an adjuvant are: 5-fluorouracil (5-FU) plus levamisole, 5FU plus

leucovorin, but also the chemotherapy with oxaliplatin and irinotecan is very common [Lee et al., 2012]. The development of new targeted drugs, such as monoclonal antibodies (mAb) against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) seem to add further benefits to patients with metastatic CRC [Garret 2011].

A kind of targeted therapy is a targeted drug delivery system that seeks to concentrate anti-cancer agents at the cancer tissue, reducing the relative concentration of the medication in the proximal tissue. For example, polymeric micelles increase the accumulation of drugs in tumor tissues utilizing the enhanced permeability and retention effect [Matsumura et al., 2008]. The main advantages of micelle are the possibility to modify their diameter, to be sure that they not pass in normal vessel walls, and to incorporate a variety of drugs.

The chemoprevention of CRC is another strategy to reducing the morbidity and the mortality from this disease; indeed, CRC has a natural history of transition from normal crypts through adenoma to overt a carcinoma, that could be arise in a time frame of 10-20 years, providing a window of opportunity for effective intervention and prevention; in addition, the occurrence and recurrence of CRC after treatment is still very high. Chemoprevention is defined as the use of specific natural or synthetic chemical agents to reverse, suppress or prevent the transition from adenoma to invasive CRC. It has been shown that chronic intake of traditional non-steroidal anti-inflammatory drugs could reduce the incidence of CRC [Klampfer et al., 2011, Hawk et al., 2003]. In addition, cyclooxygenase-2 (COX-2) inhibitors have been approved by Food and Drugs Administration (FDA), following the evidence that they are effective in reducing CRC in animal models and FAP patients [Bakhle et al., 2001]. Chemoprevention is thus of particular importance to genetically

predisposed patients and to those patients who are especially susceptible to the environmental causes of CRC [Tanaka et al., 1997 and 2009]. A lot of other pharmacological and non-pharmacological agents have been analyzed to define their potential as chemiopreventive agents for CRC [Gwyn et al., 2002]; among them, there are irreversible inhibitor of ornithine decarboxylase, calcium, folate, vitamins selenium and its derivates, and dietary fiber. Even if the exact action mechanisms of these agents are not fully understood, five potential pathways and mechanisms of these composts have been illustrated: a) increased sensitivity of cancerous cells to apoptosis, b) inhibition of angiogenesis, c) modulation of inflammation and immune response, d) decreased metastasis and e) inhibition and removal of endogen carcinogen formation *in vivo* [Hawk et al., 2003, Dempke et al., 2001, Prescott et al., 2000, Subbaramaiah et al., 2003]. The impact of these molecules could improve the treatment and the prevention of CRC.

1.2 Cancer immunotherapy

The discovery that the cells of the immune system can help fighting against cancer does the groundwork for the development of immunotherapeutic approaches. As is known, people with weakened immune system are more likely to get certain cancers. Anyway, also people with normal immune system can develop cancer when the immune system fail to recognize cancer cells as foreign agents, or when the cancer cells do not express their antigens which are different from these of normal cells. In other cases the response of the immune system can be detected, but it is often too weak to induce apoptosis in cancer cells, or the cancer cells themselves may also secret immunosuppressants that may keep the immune system in check [Mishra et al., 2013]. For these reasons, one of the main topic of researchers was, and is still today, designing a way to help the immune

system to recognize cancer cells and strengthen its response so that it can destroy the cancer cells.

1.2.1 A historical overview on cancer immunotherapy

Several discoveries have accompanied the development of immunotherapy as new branch of medicine for cancer treatments. Without mentioning the main immunological findings and without going too far back in time, we take the discovery in 1976 of recombinant human Interleukin 2 (rh IL-2) as T-cell growth factor *in vitro* [Morgan et al., 1976] the starting point of our historical travel in this topic. In 1985 it was demonstrated that the administration of rh IL-2 to patients could mediate the regression of invasive human cancer, providing the first evidence that the manipulation of human immune system could drive to tumor regression [Rosenberg et al., 1985]. This encouraging data have determined the FDA acceptance of rh IL-2 as drug for the treatment of patients with metastatic RCC and metastatic melanoma, the most immunogenic human tumors, in 1992 and 1998 respectively [Smith et al., 2008]. The discover of the first tumor antigen in 1991 [Van der Bruggen et al., 1991] leads off to the description of hundreds of antigens and antigenic epitopes expressed on cancer cells and recognized by the immune system [Rosenberg et al., 1999, Robbins et al., 2000] in the next two decades, with the aim to change over a specific immunotherapeutic approaches. These have led to a myriad of clinical trials assessing immunization with peptides, proteins, dendritic cells (DCs), recombinant viruses, whole cells and plasmidic DNA; most of them have demonstrated clinical benefits for patients, as for example the results obtained by Kantoff [Kantoff et al., 2010] after the immunization of prostate cancer patients with DCs. The difficulties observed to obtain efficient vaccines, including the inability to generate *in vivo* a large number of antitumor T-cells with high affinity for tumor antigen, as well as the potential immunosuppressant

function of tumor microenvironment [Rosenberg et al., 2005], have lead to develop other immunotherapeutic approaches. Indeed, some of that obstacles can be overlap by the use of adoptive cellular therapy (ACT), based on the administration of antitumor immune cell in patients with metastatic cancers and there are best evidences of its success [Dudley et al., 2002 and 2008 Rosenberg et al., 2008]. Although the more comforting results were obtained on melanoma patients, there are promising evidence of this approach also for other kinds of malignancies such as: EBV-induced lymphoma [Rooney et al., 1998], nasopharyngeal carcinoma [Straathof et al., 2005(a)], neuroblastoma [Pule et al., 2008], metastatic synovial sarcoma [Robbins et al., 2011] and metastatic CRC [Parkhuirst et al., 2011].

1.2.2 Immunesurveillance against tumor

The human organism has a number of different types of immune cells to effectively deal with transformed cells and foreign invaders such as viruses or other microorganisms. The cells mainly involved in this process, called “immunosurveillance”, are the T-lymphocytes, able to destroy transformed or infected body cells.

The immunosurveillance theory was formulated for the first time by Burnet in 1970 [Burnet, 1970]. Burned postulated that the development of T-cells mediated immunity during evolution was specific for the elimination of transformed cells and that T-cells continuously survey the body and eliminate those cells that underwent cancerous transformation. After several years, new studies reinvigorated and validated the immunosurveillance concept [Smyth et al., 2001, Dunn et al., 2002, Dunn et al., 2004]; this theory was supported also by Schreiber and colleagues [Schreiber et al., 2004] who have been demonstrated the direct role of lymphocytes and of the cytokine Interferon γ (IFN-γ) in tumor suppression.

It is now clear that T-cells are efficient at eliminating virus-induced malignancies, but the response to chemical or physical carcinogen-induced tumors, as it has been shown in animal models, is much weaker. This difference in response is primarily related to the nature of the antigen. The transformed cells must have phenotypical changes to be recognized as non-self by T-cells. In addition, even if the changes had occurred, a lot of mechanisms to evade the immune system could put in place. As reported by Vera and coworkers [Vera et al., 2009], the tumor immune evasion strategies include:

a) secretion of immunosuppressive cytokines such as Interleukin-10 (IL-10), Interleukin-13 (IL-13) and transforming growth factor beta (TGF-β), able to inhibit the effector T-cells [Zou et al., 2005];
b) expression's modulation of major histocompatibility complex (MHC) molecules and co-stimolatory molecules to prevent antigen-specific T-cells recognition [Zou et al., 2005];
c) recruitment of regulatory T-cells (T regs) that could inhibit the T effectors by direct contact or by secretion of soluble factors [Woo et al., 2001, Curiel et al., 2004, Marshall et al., 2004];
d) constitutive expression of an enzyme that prevents the lymphocytes proliferation, called indoleamine 2,3-dioxygenase (IDO) [Zamanakou et al., 2007];
e) apoptosis of activated T-cells through the expression of Fas ligand (FasL) [Igney et al., 2002];
f) expression of inhibitory cell surface molecules such as program death ligand (PD-L1), which interacts with PD-1 expressed on activated T-cells, and induces T-cell exhaustion [Keir et al., 2008, Dotti, 2009].

So that, the immunosurveillance appears as only one dimension of the complex relationship between the immune system and cancer [Dunn et al., 2002 and 2004, Schreiber et al., 2004]. The evidences that the immune system may also promote the emergence of primary tumors with reduced immunogenicity, able to escape immune recognition and destruction [Shankaran et al., 2001] do the groundwork for the cancer immunoediting hypothesis [Dunn et al., 2002, and 2004].

As clearly reviewed by Dunn and colleagues in 2004, cancer immune-editing is a dynamic process composed of three phases: elimination, equilibrium and escape, in which elimination represents the canonic phase of immunosurveillance, equilibrium is the period of immune-mediated latency after an incomplete tumor destruction in the elimination phase, and escape refers to the final out-growth of tumors that have outstripped immunological restraints of the equilibrium phase (Figure 3).

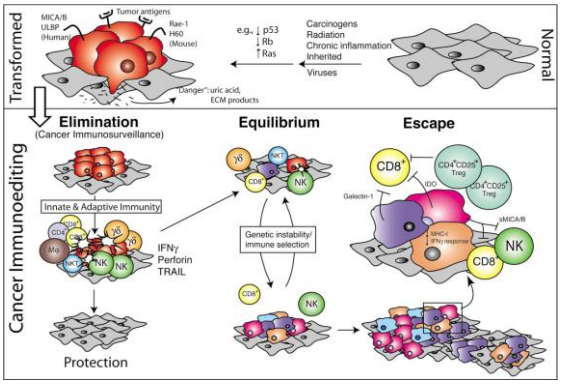


Fig. 3: The Three Phases of the Cancer Immunoediting Process [Dunn 2004]

1.2.3 Role of dendritic cells and t cd8+ lymphocytes in cancer immunotherapy

The cells of the immune system on which we have focused our attention are: the most important category of antigen presenting cells (APCs), the

DCs [Banchereau et al., 1998], and the main cellular type responsible of an anti-tumor response, the T $CD8^+$ lymphocytes.

DCs: are the most potent APCs, essential for the initiation and maintenance of immune response [Hart et al., 1997, Gallucci et al., 1999, Steinman, 1991]. DCs work at the interface between peripheral tissues and lymphoid organs. So these cells can be found in many tissues of the human body, as a heterogeneous population of specialized APCs, circulating via the blood to most of tissue [Steinman et al., 1973].

DCs can be divided in two peripheral blood (PB) DC subsets: 1) lymphoid derived DCs, that are CD11c negative and usually express high levels of CD123; 2) myeloid derived DCs, that have the opposite surface marker expression ($CD11c^+$, $CD123^-$) [Shortman et al., 2002]. DCs surface antigen expression, morphology and function vary in association with their maturation or activation status in a given tissue [Hart et al., 1997]. This is clearly evident in the circulation: whereas myeloid DCs are adept at antigen uptake, they haven't the typical "dendritic" morphology when freshly isolated and they require a period in culture before acquiring the appropriate costimulatory molecules required for an optimal T-cell stimulation [Upham et al., 2000].

However, DCs have a low concentration in PB (0,01%) and it is very hard to identify of a single DC-specific cell marker, so their isolation is very difficult from this biological fluid [Syme et al., 2005].

Anyway, in physiological conditions, DCs are present in two maturation states: immature DCs (iDCs), whose function is to capture, process and presenting antigens [Elkord et al., 2005, Brossart et al., 1997, Cella et al., 1997], and mature DCs (mDCs), which can drive T-cell clonal expansion through cell-cell interaction and cytokines production [Elkord et al., 2005,

Cella et al., 1997, Steinman et al., 1991, Macatonia et al., 1995, Koch et al., 1996, Caux et al., 1994].

Immature DCs, derived from bone marrow precursors check the extracellular milieu of peripheral tissues for the presence of pathogens and danger signals. After the recognizing of these, DCs became mature by pathogen-associated molecular patterns (PAMPs) *via* Toll like receptors (TLRs), and by inflammatory cytokines and prostaglandins released in the environment. The DCs maturation is essential for their rapidly migration to the afferent lymph nodes, that could be done following a chemokine gradient; at least mDCs are able to prime the immune response through interaction between CD40 (expressed by DCs) and CD40-ligand (expressed by the T-cells) [Nencioni et al., 2008].

Nowadays it's clear that DCs play a critical role in the induction of protective and therapeutic anti-tumor immunity, by contributing to the generation and proliferation of T Cytotoxic lymphocytes (CTLs) and helper T-cells (Th cells) [Yasuda et al., 2006]. Studies on murine models have been analyzed different strategies to induce an efficient anti-tumor immunity with tumor antigen-loaded DCs [Boczkowski et al., 1996, Fields et al., 1998, Gong et al., 1997, Mayordomo et al., 1995], that have been reported in trials in tumor-bearing patients [Hsu et al., 1996, Nestle et al., 1998, Murphy et al., 1999, Kugler et al., 2000]. The strategies used for transfer antigens from tumors to DCs for MHC class I presentation, mainly derived from *in vitro* experiments, and as reviewed by Melief include : 1) antigen from dead cells (apoptotic or necrotic tumor cells); 2) soluble antigens bound to heat shock protein (HSP); 3) soluble protein; 4) antigen-carrying vescicles secreted by some tumor cells called exosomes; 5) transfer of small antigenic protein fragments, and two others that involve the direct transfer of peptide MHC complex to DCs [Melief, 2008]. The first

mechanism has been chosen by us to develop an immunotherapeutic approach in CRC.

T $CD8^+$ lymphocytes: Once activated, these cells are called CTLs and are able to directly damage target cells by using at least three distinct effector functions: 1) release of preformed toxic substances, such as perforin, granzymes, and granulolysin; 2) triggering of programmed cell death, or apoptosis, by engagement of Fas receptor with FasL; 3) secretion of cytokines such as tumor necrosis factor alpha (TNF-α) and IFN-γ [Fragoso et al., 2002].

IFN-γ is the typical cytokine with opposing effects on tumors. It has been demonstrated that endogenously produced IFN-γ protects the host against the growth of transplanted tumors and also the formation of primary chemically induced and spontaneous tumors [Dighe et al., 1994, Kaplan et al., 1998, Shankaran et al., 2001, Street et al., 2001 and 2002]. It is an inflammatory cytokine that, for example, was up-regulated following radiation in a melanoma model [Lugade et al., 2008], and also in other cases it has conventionally been had an antagonistic effect on tumor growth [Zaidi et al., 2011], even if many studies has investigated a possible role of this cytokine in immunosuppression [Taniguchi et al., 1987, Gorbacheva et al., 2002, Katz et al., 2008, Prendergast, 2008, Ostrand-Rosenberg et al., 2009]. However, in a very recent study of Gerber and colleagues [Gerber et al., 2013], it was shown that IFN-γ is not only beneficial, but essential in mediating the antitumor effects of radiation in a mouse colon adenocarcinoma tumor. The levels of IFN-γ increased in tumor environment after irradiation and it was confirmed that cytokine production was done by $CD8^+$ T lymphocytes. Indeed, elimination of $CD8^+$ T-cells not only greatly reduce the intratumoral concentration of IFN-γ, but also abrogated any antitumor effect of radiation. The finally consideration of

Gerber et al was that although the cytokine had no direct effect on tumor cells in promoting tumor control, IFN-γ enhance the cytolytic capacity of T-cells, possibly in an autocrine manner, resulting in a decrease of tumor burden [Gerber et al., 2013].
The secretion of IFN-γ was our main parameter to evaluate a specific CTLs antitumor response.

1.2.4 Tumor antigens: features and classification

In the last decades, different studies focused their attention on the definition and classification of tumor antigens.
Two class of non-viral tumor antigen have been identified: 1) tumor-specific antigen (TSA), that are caused by somatic mutations, able to alter self-proteins through single amino acid substitutions, truncation, deletion or fusion to other protein; these antigens are expressed by autologous cancer cells but not by autologous normal control tissues or by allogenic cancer cells, so they are truly tumor specific [Oettgen et al., 1990]; 2) tumor-associated shared antigens (TAA) that result from an aberrant expression of non-mutated proteins [Schreiber et al., 2003, Dermime et al., 2004]; they are found on autologous and allogenic cancer cells as well on a subset of normal cells [Oettgen et al., 1990].
TSA have two specific advantages compared to TAA: the first is their exclusively expression on cancer cells, minimizing the risk of autoimmune destruction. Additionally there is no neonatal or peripheral tolerance to these antigens prior to tumor development, since the immune system has not been previously exposed to them. The second is that many of these TSA results in mutated proteins essential for the tumorigenic process. It means that those proteins, required to maintain the malignant phenotype, are less likely to get lost during tumor progression, even under the selective pressure of anti-cancer treatment, such as cancer immunotherapy [Sensi et

al., 2005 and 2006]. Conversely, targeting TAA, that are not essential in tumor development, could determine the formation of antigen loss variants [Lozupone et al., 2003, Maeurer et al., 1996, Jager et al., 1996, Yee et al., 2002].

Several works have been demonstrated that there is a sort of hierarchical immune response in the hosts: TSA were found to be immunodominant, while TAA were defined as immunorecessive. The disadvantage of TAA is that they could induce an immune response only when the immune dominant antigens are lost [Wortzel et al., 1983, Dudley et al., 1996].

TSA are the ideal targets of anti-cancer therapy for the reasons listed beforehand, but targeting these antigens would determine a much expensive and personalized therapy for patients, so researchers are making an effort to identify molecules that could be expressed not only from one patient with a specific kind of tumor, but from several tumors.

A third class of tumor antigens was identified, through serological investigations: these antigens are cell surface molecules widely expressed by normal or non-malignanT-cells, and their precise nature or biochemical origin is elusive [Furukawa et al., 1989]

The progresses of several molecular technologies, such as serological analysis of recombinant cDNA expression library (SEREX), microarray technologies etc., allowed the identification of many tumor antigens.

In the review of Schietinger and colleagues [Schietinger et al., 2008], TAA were subdivided in four categories, according to their expression pattern:

1) *Oncospermatogonal antigens* (cancer-testis antigens or CTA): cancer cells express these antigens that are normally found on spermatocyts/spermatogonia [such as melanoma associated antigen (MAGE) and antigens derived from the *GAGE, BAGE* and

NY-ESO-1 genes], but also on fetal ovary [Koslowski et al., 2004] and sometimes in placental trophoblasts [Jungbluth et al., 2007]. CTA are found variably in a range of cancers, including about 90% of melanomas [Zendman et al., 2003] and melanoma associated antigen A1 (MAGEA1) became the prototype of this class of molecules with a highly restricted tissue expression [Simpson et al., 2005].

2) *Differentiation antigens*: are molecules expressed on non-malignant cells of the same cell lineage as the tumor [tyrosinase related protein-1 (TRP-1), glycoprotein 100 (gp100), melanoma antigen recognized by T-cells 1 (MART-1), CD20 and epithelial cell adhesion molecule (EpCAM)];
3) *Oncofetal antigens*: these antigens can be found on embryonic and fetal tissue as well as as certains cancers [alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and 5T4]
4) *Over-expressed antigens*: are normal proteins whose expression is up-regulated in cancer cells [prostate specific antigen (PSA), wilde type p53, human epidermal growth factor receptor 2 (Her2/Neu) and EGFR]

Then usually, the expression of tumor antigens on cancer cells permits T-cells to recognize, bind and destroy tumor cells.

The vast majority of T lymphocytes express on their surface a T-cell receptor (TCR), able to bind a molecular complex consisting of an antigenic peptide bound to a molecule of the MHC on the APC. The nature of peptides could be either cellular (endogenous antigens) or derived from extracellular proteins (exogenous antigens) after a proteolytic degradation process. The function of MHC molecule is to present peptides to T-cells; there are two class of MHC molecules: MHC I binds endogenous derived

peptides, while MHC II binds exogenous derived peptides. However, through a process defined cross-priming, also exogenous antigens could be presented in association with MHC I molecule on professional APCs.
MHC II molecules are expressed only on certain cells, most of which are bone marrow derived cells, and are recognized by T $CD4^+$ helper cells, while MHC I molecules are expressed virtually on all nucleated cells and are recognized by T $CD8^+$ lymphocytes. The co-receptors CD4 and CD8 bind non-polymorfic regions of MHC molecules, whereas the TCR interacts with both MHC and their bound peptide.
Accordingly, when naïve T $CD8^+$ and T $CD4^+$ cells recognize tumor antigen presented in association with MHC I and MHC II molecules respectively, and when the essential costimulatory signals are provided, these cells become activated.
Briefly, activated T $CD4^+$ cells secrete cytokines that regulate activated T $CD8^+$ cells, but cannot interact with cells that not express MHC II molecules; conversely T $CD8^+$ cells can directly interact with MHC I expressing tumor cells and mediate the lyse of tumor cells after this recognition [Goedegebuure et al., 2002].

1.2.5 Immunotherapeutic approaches in cancer

On the basis of what was previously decribed, immunotherapy can be divided in three main categories, as exhaustively showed in a review of Rosemberg and colleagues [Rosenberg et al., 2008]:
1) *Non specific immunomodulation*: includes the administration of rh IL-2 in order to activate endogenous reactive T-cells *in vivo*, causing regression of many human cancers [Morgan et al., 2006, Rosemberg et al., 1985]. The disadvantage of this treatment is the toxicity of the cytokine if administrated at high doses. Another non specific immunomodulation include the use of mAbs direct against the cytotoxic T-lymphocytes-associated 4 (CTLA4), a

cell surface inhibitory molecule; as in the case of rh IL-2, the best clinical responses are those of melanoma and RCC patients [Phan et al., 2003, Attia et al., 2005, Margolin et al., 2000].

2) *Active immunotherapy*: includes cancer vaccines, that are based on immunizing cancer patients against their autologous cancers using either whole cells, peptides or a wide variety of immunizing vectors. This idea behind cancer vaccines is generally meant to boost the immune system to fight against the cancer just like vaccine to infection. Cancer vaccines are active immunotherapy because they triggering the patient's immune system to respond. However, they could be divided in preventive and therapeutic cancer vaccines. Although the concept of preventive vaccine is very intriguing, and there are some examples such as the vaccines against different genotypes of human papillomavirus, in order to prevent the tumor of uterine cervice, the most of cancer vaccine in clinical trials are therapeutic. The first therapeutic vaccine, approved in 2010 by the FDA, was called Provenge and its successful for prostate cancer [Brower et al., 2010].

Cancer vaccines typically consist of a source of cancer-associated material (antigen), along with other components, such as adjuvants, to further increase the specific antitumor immune response. Few example of cancer vaccines include: tumor cell vaccines, antigen vaccines, DC vaccines (like Provenge), anti-idiotype vaccines, DNA vaccines, and vector-based vaccines [Mishra et al., 2013]. One of the main disadvantages of cancer vaccines is that, if the tumor cells mutate after chemotherapy or radiation treatment, changing the targets of the vaccine, they become ineffective.

3) *ACT*: is also called passive immunotherapy and refers to antibodies, or more frequent other immune-system components that are made outside of the body, in laboratory, and administered to patients to provide immunity

against cancer, with no active immune stimulation that is conversely produced in patients by cancer vaccines.

Usually, after the identification *ex vivo* of autologous or allogenic lymphocytes with antitumor activity, they are re-infused into cancer patients, often in association with growth factors to promote their expansion and survival *in vivo*. The advantages of this approach include the necessity to identify only a small number of anti-tumor specific T lymphocytes with the appropriate characteristics, since they can be expanded to large number *ex vivo* before treatments; in addition, through *in vitro* test, the exact population of interest, able to mediate cancer regression, could be easily identified and selected for expansion; another important advantage is that these cells can be activated in laboratory in absolute absence of endogenous inhibitory factors and thus can be induced to exhibit the required anti-tumor effector function [Rosenberg et al., 2008].

1.2.5.1 Adoptive cell therapy using tumor infiltrating lymphocyte (TIL)

One of the possible ACT strategies is based on the finding in 1987 that T infiltrating lymphocytes (TIL) could be isolated from melanoma tumors and cultured *in vitro* in presence of rh IL-2. These cells exhibit MHC-restricted recognition of autologous melanoma [Muul et al., 1987]. It has been demonstrated that, improving culture methods, about 10^{11} TIL could be generate having a specific melanoma activity in 81% of 36 consecutive patients [Dudley et al., 2003]. The infusion of TIL grown from the resected nodules of metastatic melanoma patients represent the clearest successful example of ACT for the treatment of patients with metastatic solid cancer, even if the first effective of ACT was demonstrated in the clinical context of haematopoietic malignancy as reported by Rosemberg and colleagues in 1988 [Rosenberg et al., 1988]. However, the main disadvantage of their clinical trial was the short persistence of the transferred cells *in vivo*. To

overlap this limit, a profound host lymphodepletion is necessary before cell transfer [Dudley et al., 2002 and 2005, Gattinoni et al., 2005]. Adverse effects in the lymphodepletion trial included opportunistic infections an the frequent induction of vitiligo and uveitis, presumably due to autoimmunity.
Other clinical trials that used ACT based on TIL re-infusion were shown in a review of June [June et al., 2007], in which also the different strategies are reported to augment ACT. These randomized clinical trials involved several kind of tumors, like gastric cancer [Kono et al., 2002], renal cancer [Figlin et al., 1999], lung cancer [Ratto et al., 1996], and still melanoma [Dreno et al., 2002]. Several studies used as main antitumor cells not TIL, but CTLs derived from PB. Also in this case the best clinical result of this strategy is melanoma tumor: these cells were used to treat patients with refractory , metastatic melanoma and 8 of the 20 patients had minor, mixed or stable antitumor immune responses [Yee et al., 2002]. It has been demonstrated in a previously work of Yee and coworker [Yee et al., 2000] that the infusion of autologous MART-1-specific T $CD8^+$ cells in to a patients with metastatic melanoma resulted in T-cell infiltration in both the skin and tumor tissue. The destruction of the normal melanocytes and the outgrowth of MART-1 negative tumor demonstrated both the *in vivo* efficacy of the infused T-cell population and the selection of tumor variant with loss of MART-1 expression [Yee et al., 2000]. However, this problem can be overtaken infusing CTLs clones with multiple antigenic specificity. An additional tumor in which PB T-cell were activated *in vitro* and re-infused in patients is the hepatocellular carcinoma [Takayama et al., 2000].

1.2.5.2 Adoptive cell therapy for cancer patients expressing viral antigen or alloantigens

As mentioned before, ACT is an effective treatment for several haematopoietic malignancy. It clear that when the target antigen on a tumor

is foreign to the host, as the viral antigen or alloantigens, the avidity of T-cell is more higher than in other situations and the destruction of tumors can be very large. In 1990 Kolb and colleagues have shown that the treatment of three relapsed chronic myeloid leukaemia patients with buffy coats cells from hematopoietic stem cell (HSC) bone marrow donor determined cytogenetic remission (Kolb et al., 1990). Also in patients with relapsed chronic myeloid leukaemia the infusion of donor lymphocytes could mediate molecular remission in 70-80% of cases, and in a minority of patients with relapsed multiple myeloma, following treatment with allogenic HSC transplantation [Mackinnon et al., 1995].

The adoptive transfer of EBV-specific T-cell lines and CTLs for the therapy of EBV-induced lymphomas is perhaps the best demonstration of clinically efficacious of ACT [Heslop et al., 1997, O'Reilly et al., 1997]. These lymphomas express latent EBV antigens, including the immunodominant EBV nuclear antigens (EBNA) EBNA-3A, B and C, that are ideal targets for immunotherapy. The risk for patients received allogenic HSC transplants in combination with immunosoppressive drugs to avoid episodes of graft versus host diseases is the developing of post-trasplant lympoproliferative disease (PTLD). In a study of Rooney et al. it has been demonstrated that donor-derived EBV CTL lines infused in 60 patients with a high risk to develop PTLD established the onset of malignancy in none of them, compared with the 11,5% of historical controls [Rooney et al., 1995]. This strategy was used of for the treatment of 16 nasopharyngeal carcinoma patients at stage 4, refractory to conventional treatments and in 11 patients with Hodgkin disease. It was possible since poorly differentiated nasopharyngeal carcinoma and Hodgkin's lymphoma express the EBV latent membrane proteins (LMP-1,2), as well as the EBV EBNA-1 antigen. The results of these studies have shown two complete remission and three

partial remission in the context of nasopharyngeal carcinoma and three objective responses in Hodgkin's lymphoma patients [Comoli et al., 2005, Bollard et al., 2004].

These results underlined as the administration of an avid anti-tumor cell, targeting a highly expressed antigen can result in cancer regression. Strength of the effectiveness of ACT directed against EBV and allogenic antigens, as well as the prevention of Cytomegalovirus (CMV) infection in immunosuppressed patients after the infusion of CMV-reactive autologous T-cell lines [Riddell et al., 1991], researchers have begun to evaluate all these data in order to improve the ACT approaches for the treatment of solid tumor.

1.2.5.3 Adoptive cell therapy using gene modified lymphocytes

The evidences that TIL with high avidity for tumor antigens could be generated essentially starting from only melanoma specimens, and the need to produce antitumor T-cells with broad reactivity against shared cancer-associated antigens expressed on different kind of tumors, have done the groundwork for the introducing of gene modified lymphocytes in ACT. This strategy is very useful also to try to ride over the mechanisms of immune evasion that mitigate the tumor-directed T-cell reactivity.

Genetic modification of T-cells can be divided in such groups: one of the most important is represented by the genetically modified T-cells to allow them to recognize antigens expressed by tumor cells that could be done inducing expression of constitutive androstane (CAR) receptors that recognize tumors through single-chian variable fragments (scFv) isolated from specific antibodies [Eshhar et al., 1993] and inducing gene modification with α and β TCR chains cloned from TAA-specific T-cell clones with high antigen avidity [Schumacher, 2002]

Other genetic modifications were carried out to improve T-cell proliferation and survival: these includes genetically modification of T-cells with retroviral vectors encoding IL-2 or Interleukin-15 (IL-15) to produce cells which are cytokine self-sufficient and self-sustaining [Quintarelli et al., 2007, Liu et al., 2001, Hsu et al., 2005], preventing in this way also the common side effects of high doses of rh IL-2; Quintarelli and colleagues have shown that these genetically modified T-cells had enhanced persistence and superior anti-tumor activity *in vivo* compared to the unmodified cells [Quintarelli et al., 2007]. Since T-cell proliferation requires continued antigenic stimulation, either *via* direct interaction with tumor cells or through professional APCs that cross present tumor antigens, and since many tumors lack expression of co-stimulatory molecules, such as CD80 and CD86, could be important to force the expression of co-stimulatory ligands [Stephan et al., 2007]. A way to increase the survival of T-cells is represented by their trasduction with anti-apoptotic genes, such as B cell lymphoma gene-2 (*Bcl-2*) and B cell lymphoma gene-extra-large (*Bcl-xL*) [Charo et al., 2005]. In addition, strategies to make antigen specific CTLs resistant to the effects of immunosuppressive drugs, in order to prevent the onset of virus-associated complication such as EBV-post transplant lymphoma [Paya et al., 1999], have been recently developed using the small interference RNA (siRNA) technology [De Angelis et al., 2009, Brein et al., 2009].

It is important to be successful in counteract the immunosuppressive tumor microenvironment; since the main tumor evasion strategy is the local secretion of TGF-β, researcher have developed, in murine models, a way to modify CTLs, supporting their expression of dominant negative TGF-β receptor type II: the results are encouraging because these modified CTLs are resistant to the antiproliferative effect of TGF- β both *in vitro* and *in vivo*

[Bollard et al., 2002]; a clinical trial based on this strategy is on going for the treatment of patients with relapsed/refractory Hodgkin's lymphoma.

Some genetic modifications were designed to redirect T-cell migration *in vivo*, because tumors can generate chemokine milieu that significantly modifies the trafficking of Th1, Th2 cells and T regs [Dilloo et al., 1996, Gao et al., 2008], unbalancing dangerously to the last two.

Finally, other modifications were done to reduce the inevitable risk of toxicity and of unwanted proliferation of the genetically modified produced T-cells. To allow the rapid and complete elimination of infused cells, several groups have evaluated safety switches or suicide genes, which can be triggered when toxicity occur [Ciceri et al., 2009, Traversari et al., 2007, Thomis et al., 2001, Straathof et al., 2005 (b), Tey et al., 2007].

1.3 Immunotherapy in CRC

Nowadays immunotherapy for CRC remains only and experimental option, although some clinical trials of cancer immunotherapy have demonstrated a potential benefit for these patients.

As mentioned previously, immunotherapy has the potential to eradicate cancer by eliciting immune responses through the recognition of specific antigens on tumor cells. However, the lack of antigens that are effectively tumor-specific limits the development of immunotherapy. This consideration is especially true for CRC, that is a very poorly immunogenic tumor.

1.3.1 The tumor antigens of CRC

For CRC patients, the evidence that tumor cells are able to induce a tumor-specific T-cell response in the autologous setting is still scarce, even if few immunological studies have been performed [Dalerba et al., 2003].

On the bases of melanoma immunotherapeutic results, the first investigations in CRC were done using TIL, but due to difficulties in

obtaining stable CRC cell lines, encountered also in our project, only few *in vitro* studies have demonstrated the human leukocyte (HLA)-restricted anti-tumor reactivity of TIL obtained from colorectal carcinoma lesions. This is one of the reasons because few molecular defined TAA has been reported for CRC.

Through this approach, Saeterdal and coworkers [Saeterdal et al., 2001] have been identified a peptide derived from a frameshift mutation of the transforming growth factor β receptor type II (TGFβRII) and presented by HLA-DR, that is able to be recognized by TIL purified from CRC lesions and expanded *in vitro* by rh IL-2. Since the mutated form of TGFβRII is detectable only in tumors, and its inactivation occurs in 90% of microsatellite instability (MSI) CRC, it could be considered as a TSA and could be a good candidates for immunotherapy, also because TGFβRII gene appears to play an active role in tumorigenesis. After that, other two peptides were identified with this strategy: squamous cell carcinoma antigen recognized by T-cell 3 (SART-3) and cyclophilin B (CycB) [Ito et al., 2001, Tamura et al., 1999, Yang et al., 1999, Myagi et al., 2001]. The first protein is more promising than the second, that is ubiquitously expressed in normal as well as in cancer cells; in fact SART-3 was used, in combination with different doses of SART-3 derivate peptides, for phase I clinical trial and results showed a significant increase in the precursor frequency of peptide-specific T-cells in peripheral blood mononuclear cells (PBMCs) after three cycles of vaccination, even if without objective clinical responses.

Since a specific antitumor T-cell activity is not only local (detected in tumor site) but also systemic, different strategies, based on a reverse immunological approach using the properties of DCs, that could be load also with a tumor lysates and so without the need to establish molecular

defined antigens, have been developed. Bremers and colleagues [Bremers et al., 2000] showed that tumor specific T-cells, generated after the PBMC stimulation with DCs loaded with tumor lysate, are directed against TAA epitopes derived either from differentiation or from TSA. Consequently the presence of a relative high precursors frequency of TAA-specific T-cells in PB of the immunized patients, and an *in vivo* systemic immunisation, occurred during the natural course of disease, could be hypothesised [Dalerba et al., 2003].

Through this second approach several epitopes, able to induce a spontaneous systemic immunity in CRC, have been molecularly identified. Among them, an important role is played by CEA, the most old studied molecule [Gold et al., 1965], EpCAM and Her-2/neu proteins, belonging to the category of differentiation antigens. The first two are cell surphace adhesion molecules expressed by more than 90% of CRC, while Her-2/neu is an epithelial growth factor receptor over-expressed in a subset of CRC.

In particularly, it has been demonstrated that CEA promote the aggregation of CRC cells and also may facilitate metastasis by acting as L-selectin and E-selectin ligands [Thomas et al., 2008]. It is expressed by several adult tissues and can be found at low levels in healthy adult blood [Benchimol 1989, Thomas 2008], but it is over-expressed by adenocarcinomas of the colon, rectum, breast and lung and can be detected at high levels in the serum of these patients [Hammarstrom, 1999]. These features make CEA as diagnostic marker, but its expression also in normal tissues limits the specific immune responses elicited by cancer vaccines [Xiang et al., 2013].

Another potential TAA expressed by CRC is $p56^{lck}$, a tyrosine kinase member of the src family, essential for T-cell development and function, potentially involved in the process of neoplastic transformation and progression [McCracken et al., 1997]. Similarly with the data on anti-CEA,

anti-EpCAMand anti-Her-2/neu T-cells, CTLs frequency against HLA-restricted $p56^{lck}$ peptides reached a detectable levels only in advanced CRC stages [Harashima et al., 2001, Imai et al., 2001].

Two other potential antigens for CRC immunotherapy are mucin 1 (MUC1) and Guanylyl cyclase C (GCC). MUC1 is a transmembrane glycoprotein founded on the apical surface of secretory epithelial cells [Hollingsworth et al., 2004], able to bind pathogens to limit bacterial invasion but also to regulate cell motility and survival [Linden et al., 2009; Singh et al., 2006]. It could be over-expressed in several types of adenocarcinoma, and specifically in CRC, could be also subject to an abnormal glycosylation, events that are associated with a poor prognosis prognosis by regulating tumor-promoting signaling pathways such as β-catenin and k-ras [Ajioka et al., 1997; Singh et al., 2006]. Guanilate cyclase 2 C (GUCY2C) is a receptor for the endogenous hormones guanylin and uroguanylin and exogenous bacterial heat-stable enterotoxin [Lucas et al., 2000]. It is primarily expressed on the apical surfaces of intestinal epithelial cells, regulating numerous physiological and pathophysiological processes [Kim et al., 2013] and it has been previously reported that GUCY2C expression persists through all stages of colorectal tumorigenesis from premalignant polyps to distant CRC metastases [Cagir et al., 1999; Carrithers et al., 1996; Waldman et al., 1998]. Since its expression it has been shown to be maintained in the 95% of metastatic CRC [Carrithers et al., 1996, Schulz et al., 2006], GUCY2C could be used as biomarker for metastatic CRC, as confirmed by several retrospective and prospective clinical trials [Cagir et al., 1999; Carrithers et al., 1996, Waldman et al., 2009].

Other antigens, such as Sialyl-Tn, surviving, as well as mutated antigens, including p53 and k-ras have been studied in CRC, though without great success.

1.3.2 Active immunotherapy in CRC

As regards the specific case of CRC, although the concept of a preventative vaccine is appealing, current CRC vaccines are applied to activate the immune system to destroy tumours once they are detectable and, therefore, are considered 'therapeutic'. As mentioned previously, the vaccines could be divided in six main categories; some of these have been clearly discussed by Xiang and colleagues [Xiang et al., 2013], and those used for CRC are reported below:

1) Autologous tumor cell vaccines: the preparation of these vaccines start from tumor cells isolated from patients, engineered into a vaccine *ex vivo*, and re-administered to the patients. It is not required the definition of specific tumor antigens since whole autologous tumor cells comprise all tumor antigens, but a significant disadvantage to this approach is the difficult to generate a "universal" vaccine applicable to each patient. In addition, the immune response to these vaccines is very low, probably due to a lacking representation of specific tumor antigens in the vaccine [Lokhov et al., 2010]; in fact only a small proportion of the proteins expressed by a cancer cell are specific to tumor cells, while the vast majority of antigens in the vaccine are shared among normal cells [Xiang et al., 2013]. Nowadays, the results of this approach has not clinical benefit to patients. However, there are some clinical trials based on the use of whole tumor cell lysates in association with bacillus Calmette-Guerìn (BCG) or bacterial cell wall products as adjuvants, but the results were not statistically significant between vaccine and negative control group [Gray et al., 1989]. OncoVAX (Vaccinogen, Inc.) is a personalized antitumor vaccine using irradiated non-tumorigenic autologous tumor cells with BCG [Uyl-De Groot et al., 2005]. It has been demonstrated that in stage II of CRC this vaccine had a better outcome on survival and disease-free survival in

vaccinated patients than controls, even if the recurrence events are frequent [Harris et al., 2001].

Another strategy of CRC tumor vaccine uses autologous, virus-infected (such as with Newcastle disease virus), irradiated tumor cells, without BCG because the virus infection provides an adjuvant effect, but in this case patients had no significant improvement in overall survival, disease-free survival or metastases-free survival, even if subgroups analysis suggested some benefit from this vaccine [Schulze et al., 2009].

2) Peptide vaccines: These vaccines are based on the identification and synthesis of epitopes, which can lead an anti-tumor antigen specific immune response. Also these vaccines are often associated with adjuvants. The main advantages of this vaccine's category are the easy production and the low cost [Parmiani et al., 2002], and a low autoimmunity risk since they are produced from truly tumor specific antigens, like mutate peptides. Conversely, their major disadvantages are the cancer recurrence due to the antigenic escape, the poor immunogenicity and the HLA-restriction, that limit these vaccines to specific HLA-haplotypes [Bartnik et al., 2013, Parmiani et al., 2002]. In a study of Okuno and co-worker [Okuno et al., 2011] was analysed the clinical response of 21 CRC patients, after a peptide vaccination with two TSA: ring finger protein 43 (RNF43), and translocase of outer mitochondrial membrane 34 (TOMM34), whose expressions were up-regulated in about 80% of CRC specimens compared to normal mucosa [Shimokawa et al., 2006, Yagyu et al., 2004]; results showed that 38% and of 57% patients had a positive CTLs response against both peptides and one of them respectively, while one patient did not respond [Okuno et al., 2011]. Other complete examples of peptide vaccines could be found in a review of Merika et al. [Merika et al., 2010].

However, future phase II and III studies are needed to defined the real efficacy of these approaches compared to established treatments for CRC.
3) DC vaccines: DCs can be collected from patients, pulsed with tumor epitopes, matured *ex vivo*, and transferred back into patients as cancer vaccine to induce antitumor activity. As mentioned before, there are several ways to load DCs with tumor antigen, and these methods include: pulsing DCs with peptides derived by tumor antigens [Mayordomo et al., 1995], or tumor cell lysates [Berard et al., 2000] and physically fused DCs with tumor cells [Gong et al., 1997]. In addition, DCs may be also transduced with non-replication recombinant viral vectors, or transfected with RNA or, less commonly, plasmid vectors encoding TAA [Bonaccorsi et al., 2013]. Different clinical trials have been used this vaccination strategy in CRC. For example, tumor cell lysate pulsed DC is able to induce an antitumor T-cell response both *in vitro* and *in vivo* [Wu et al., 2010, Tamir et al., 2007]. A phase II study has reported that 20 advanced CRC patients, treated with autologous DCs pulsed with allogenic tumor cell lysate contained CTA, showing median survival of 5,3 months with stable disease in 24% patients and without toxic effects [Burgdorf et al., 2008]. Viral vectors have also been used to load DCs with tumor antigens or trasduce them with tumor specific genes such as CD40L [Liu et al., 2002], often in association with co-stimulatory molecules or cytokines, in order to enhance the T-cell response. Since CEA is the most widely used antigen for loading DCs [Morse et al., 1999], a lot of vaccination's clinical trials are based on this strategy in CRC [Itoh et al., 2002, Morse et al., 2005, Liu et al., 2004, Fong et al., 2001, Babatz et al., 2006]. Other DC vaccines have investigated the responses to multiple TAAs, loading autologous DCs with peptides derived of multiple TAA to determine priming of antigen specific T $CD8^+$ cells and

whether responses to all the antigens included in the vaccine can be raised [Hasegawa et al., 1998, Kavanagh et al., 2007].

The results of these study are encouraging in much cases, but most researches are necessary in order to improve T-cell migration and to facilitate transfer of activated CTLs to tumor sites, since at now, objective clinical responses to these vaccinations in CRC remain low.

4) DNA vaccines: a DNA vaccine is naked plasmid DNA that induces expression of specific antigens upon delivery to mammalian cells. The action mechanisms of these vaccines to activate immune system rely several processes like the interaction between the un-methylated CpG motifs of DNA plasmids derived from bacteria, and the Toll-like Receptor 9 (TLR9) on APCs, leading to DCs maturation [Hammiet et al., 2000]. The presentation of plasmid DNA can occur in a direct or indirect manner: during direct presentation plasmid DNA is delivered directly into DCs, resulting in antigen expression and presentation by the DCs. During the indirect presentation, plasmid is delivered by parenchymal cells, which express the antigens [Xiang et al., 2013]; after that, the antigen is acquired by DCs that lead the cross presentation mechanism to naïve T-cells in lymph nodes to induce adaptive immunity. However, at now no DNA vaccines for human cancer have been approved. An example for CRC is a phase I clinical study based on a DNA vaccine expressing CEA and hepatitis B surface antigen; in this trial, even if immune response was observed in 23% of metastatic CRC patients, no objective clinical responses were observed [Conry et al., 2002].

5)Viral-vector vaccines: viral vectors, that include mainly recombinant poxviruses, lentiviruses, retroviruses, adenovirus, could be engineered to express tumor antigens and the natural immunogenicity of viral vectors acts as an adjuvant to help boost tumor antigen-specific immune response; in

spite of the disadvantages based on potential pathogenesis and mutational ability, several viral-vector vaccines have been proposed for CRC in clinical trials. It is important to clarify that the majority of CEA vaccines use viruses such as ALVAC for its delivery to tissue, often in association with co-stimulatory molecules [Horig et al., 2000, von Mehren et al., 2000], but the objective clinical responses were disappointing. ALVAC has also been used as a vector to deliver EPCAM, an important mediator of cell-cell interaction and a factor involved in the growth, differentiation and organization within tissues. EPCAM functions are similar to KSA antigen, a human pancarcinoma antigen highly expressed in colon tumors. The results of Ullenhag and colleagues shown a strong IFN-γ immune response after this kind of vaccination [Ullenhag et al., 2003]. Another viral-vector vaccine is directed against the antigen MUC-1 and it seems to be promising in patients with inflammatory bowel disease (IBD), in which a strong immune response against this antigen has been observed. It will be important to clarify if this response can reverse the premalignant microenvironment, typical of these patients, and prevent the likely development of CRC.

6)Anti-idiotypic antibodies vaccines: it was demonstrated that mAbs can be used also as vaccine, exploiting the so-called Jerne's idiotypic network [Foon et al., 1999, Herlyn et al., 1996, Maxwell-Armstrong et al., 1998]. According to the idiotypic network hypothesis, a mAb directed against one specific TAA, can be used to immunise animals and induce the production of polyclonal antibodies directed against its variable region [Dalerba et al., 2003]. These antibodies could be used in turn as vaccines in order to induce a second round of anti-idiotypic antibodies that should be similar to the first antibodies, recognizing the original TAA on the patient's cancer cells. On the basis of this strategy, several clinical trials for CRC have been

started using anti-idiotypic antibodies mimicking the antigen CEA, Ep-CAM or CD55 (gp72 antigen). These antibodies induced an immunization in cancer patients, but with low clinical efficacy and their overall survival was not improved, neither with anti-idiotypic antibody against CEA [Foon et al., 1997], nor Ep-CAM [Herlyn et al., 1996, Samonigg et al., 1999], nor CD55 [Denton et al., 1994, Maxwell-Armstrong et al., 1998]. These antibodies with low clinical activity as vaccines are ongoing as adjuvant setting in other active immunization strategies [Durrant et al., 2000, Birebent et al., 2001].

1.3.3 Adoptive immunotherapy in CRC

Passive immunotherapy is the therapeutic administration to the patient of live cellular immune effectors, usually after *in vitro* expansion, activation and/or gene modification, in order to improve their anti-tumor activity. One form of this therapy is ACT. It is known that the majority of ACTs focused on T-cell therapy, due to the highly specific nature and potent killing ability of T-cells [Xiang et al., 2013]. One of the main advantages of this approach is that, with an *ex vivo* reprogramming and activation, T-cells may overcome some mechanisms of self-tolerance, which inhibit T-cell activation *in vivo* [Restifo et al., 2012]. Indeed, it has been demonstrated that the administration of large numbers of tumor specific T-cells may induce cancer regression. As described previously, disadvantages of this approach are the potential lack of immune memory, poor persistence of adoptive T-cells *in vivo*, long time (from 4 to 16 weeks) to produce these cells in research's laboratory, high costs, as well as the risk of severe side effects.

Basing on melanoma immunotherapeutic strategies, the primary approaches for ACT in CRC have used TIL, or genetically engineered T-cells, since it has been demonstrated that some tumors posses tumor-antigen-specific T-cells within tumor microenvironment [Dudley et al.,

2002], but it is known that these cells are suppressed or dysfunctional such that cancer cells overwhelm the response [Whiteside, 2006].

It should be emphasized that although melanoma is unique in its ability to naturally result in the generation of anti-tumor T-cells *in vivo*, there does not appear to be any difference in the susceptibility of different cancer types to the anti-tumor activity of lymphocytes. Thus principles that are being learned about the effectiveness of ACT in patients with melanoma can be of value in applying ACT therapy to patients with other cancer types.

Unfortunately, it seems that the use of TILs, which can be re-stimulated *ex vivo* to revert their unresponsive state, is limited to melanoma patients due to a higher immunogenicity of melanoma in comparison to other cancers.

Alternatively, the generation of genetically engineered T-cells expressing receptors, having specific affinity for tumor antigens could facilitate the targeting of virtually any tumor type. Indeed, T-cells engineered to express high avidity TCRs target tumors of various histological origins [Xiang et al., 2013]. The disadvantage of this approach is the TCR limitation to patients with the corresponding MHC haplotype. Conversely, the use of CARs, which express a scFv derived from a tumor antigen-recognizing mAb, fused to intracellular T-cell signaling domains, can be used universally across all patients since CARs target native antigens on the surface of tumors without MHC restriction [Xiang et al., 2013]. In a study of Parkhurst and colleagues [Parkhurst et al., 2011] a phase I trial was done in three metastatic CRC patients using T-cells engineered to express a high avidity CEA-specific murine TCR; although all patients showed a decreased CEA serum levels, severe side effect was observed: all three patients developed a severe transient inflammatory colitis. In another clinical trial of Morgan an co-workers [Morgan et al., 2010], one metastatic CRC patient was treated with

Her2-specific CAR T-cells, but also in this case adversely effects were observed.

However, also previous studies, in which adoptive immunotherapy for CRC has been attempted with IFN-γ activated macrophages [Lopez et al., 1992, Hennemann et al., 1995, Eymard et al., 1996], and with IL-2-activated lymphocyte effectors, such as TIL or lymphokine activated killer cells (LAK), showed no substantial clinical efficacy [Rosenberg et al., 1992, Hawkins et al., 1994, Dillman et al., 1991, Fabbri et al., 2000], probably due to the low frequency of anti-tumor T-cells in lymphocyte preparation [Parmiani, 1990] and to the escape of T-cell response by tumor cells due to HLA-loss variants [Marincola et al., 2000].

Up to now, ACT has failed to demonstrate safety and efficacy in CRC patients, so future studies will have to identify which are the mechanisms that can selectively eliminate cancer cells, without damage normal tissues, and which are the best strategies to produce a great number of activated and tumor specific T-cells.

Among the strategies employed for *in vitro* inducing an adequate activation and expansion of tumor-reactive T-cells, an actively investigated approach involve the use of DCs pulsed with whole tumor cell preparations, with either tumor extracts or apoptotic tumor cells, to cross-prime CTLs [Schnurr et al, 2002; Kurokawa et al, 2001].

A recently described procedure for generating *in vitro* large numbers of anti-tumor HLA restricted CTLs, alternative to the exploitation of TIL, involves stimulating patient's $CD8^+$ -enriched PBMCs with DCs pulsed with apoptotic solid tumor cells as a source of tumor antigens [Montagna et al, 2004]. The advantage of this experimental approach, utilizing whole tumor cells to stimulate cultures, is that it requires neither the definition of specific tumor antigens, nor the availability of TIL, thus being potentially applicable

in the generation of anti-tumor CTLs against a wide variety of solid tumors, and so even against CRC, for which a successful ACT approach was still not found.

2. AIM OF THE STUDY

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. More than 1 million people worldwide are diagnosed with CRC each year, and a staggering 0.5 million die of the disease in the same time period. The finding of new therapeutic strategies to treat CRC patients could be useful to improve these discouraging data.

The adoptive cellular therapy (ACT), based on the transfer of *ex-vivo* tumor-induced T cytotoxic lymphocytes (CTLs) provides a promising approach in cancer immunotherapy.

The *ex vivo* production of T-lymphocytes with specific reactivity towards the tumor cells is a prerequisite for designing an effective immunotherapy, aimed at controlling the tumor growth and overcoming the immune evasion mechanisms of the tumor.

It is well known that the success of ACT with *ex vivo* expanded anti-tumor CTLs depends on the number, features and abilities of the administered cells. The activation of CTLs is usually mediated by autologous dendritic cells (DCs) pulsed with either tumor extracts or apoptotic tumor cells, to cross-prime the cells. This strategy has been successfully adopted in metastatic melanoma, and in other selected solid tumors, but not in CRC, for the difficulties in isolating tumor infiltrating lymphocytes (TILs), in identifying specific or associated tumor antigens, poorly expressed in CRC, and in amplifying a sufficient quantity of autologous tumor-reactive T-cells, capable of preserving their cytotoxic capacity after expansion for therapeutic use.

The main aim of the project was the extension of the ACT approach to the treatment of CRC, by retrieving the tumor-reactive T-cells from the peripheral blood mononuclear cells (PBMCs) of the patients affected by CRC. In order to fulfil this aim, primary colon cancer cell lines were established for CRC biopsies, and induced to enter the apoptosis process.

Then, the tumor cells were cocultured in presence of autologous CTLs, generated by stimulation of patients' $CD8^+$ enriched PBMCs, and of DCs. The improvement of the ACT strategy could determine the development of a novel tumor-specific therapeutic approach in CRC treatment, characterized by increased efficacy and decreased toxicity.

3. MATERIALS AND METHODS

3.1 CRC patients enrollment

Starting from Local Ethical Committee permission and the signature of the informed consent by patients, 78 subjects affected by CRC and undergoing surgery were enrolled. Thirty-nine CRC patients specimens were obtained from San Giuseppe Hospital (Milan), and 39 from “Città Studi” Clinical Institute (Milan).

The enrollment of the patients was performed fulfilling the following inclusion criteria:

- Patients ≥18 years old.
- Patients with diagnosis of CRC.
- Possibility to obtain a fresh tumor specimen from the patient.
- Possibility to collect PB samples from the patient.

Exclusion criteria comprise:

- Absence of signed informed consent.
- Inadeguacy of samples.

SEX	N (%)	*MEAN AGE (range)*
Female	43/78 (55,1%)	75,1 (46-87)
Male	35/78 (44,9%)	70,1 (53-92)

Table 1: Case Study

3.2 Primary tumor cell lines

Tumor samples were obtained at surgery, and immediately placed in sterile RPMI 1640 medium (Euroclone) added with 20% of Fetal Bovine Serum (FBS) (South America, Euroclone), 1X L-Glutamine (Euroclone), 1X penicillin/streptomycin (Euroclone), 50 µg/mL gentamicin (Euroclone), and with 0,25 µg/mL Amphotericin B (Euroclone) to avoid infectious agents

contaminations. The medium with colon biopsy must be rapidly delivered in to the laboratory to be processed (Figure 4).

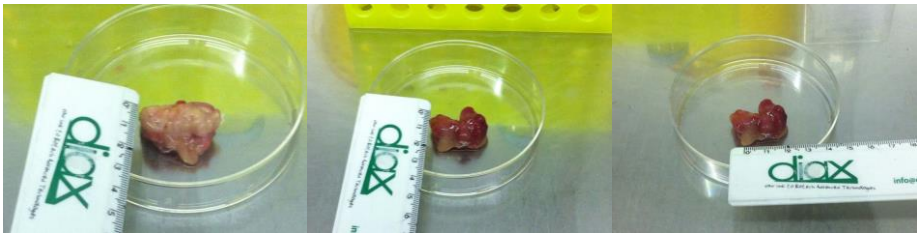


Fig.4: Colon biopsy (2cm x 2cm).

After removing fat and necrotic tissue, tumor biopsy was placed in GentleMacs Tube (Miltenyi Biotec) and dissociated to a single-cell suspension using the Gentlemacs® dissociator (Miltenyi Biotec), in accordance to manufacturer's practice. The cell suspension was then filtered using a Cell Strainer (70µm) (Beckon Dikinson) in order to keep out the tumor residues.

At first, tumor cells were seeded in 24-well at 0.5-1x10^6 cell/mL in transporting medium described before, and cultured at 37°C-5% CO_2 over a 2-3 weeks period; later RPMI medium was replaced by CellGro (CellGenix), also supplemented with 20% FBS (Euroclone) and with the same amount of antibiotics and antimycotics, to improve the obtainment of primary tumor cell lines. Anyhow, upon tissue/cell adherence, medium volume was gradually increased over 3-5 days, and the changed weekly until a substantial outgrowth of cells was observed [Montagna et al., 2004]. When tumor cells cover at least 70% of growth surface they were washed with phosphate buffered saline (PBS) (Euroclone), tripsinized and sub-cultured by 1:2 or 1:3 split ratio; tripsin (0,05%, Euroclone) is also useful to remove any fibroblast contamination from culture. Early-passage, rather than long term passage tumor cells were used to minimize the possibility of

modifications of primary characteristics as a consequence of extensive re-culturing (Freshney 1985). Tumor cell lines, after morphological-phenotypical analysis, were cryopreserved in freezing medium, composed by 90% FBS (Euroclone) and 10% Dimethyl sulfoxide (DMSO) (Euroclone), until their subsequent use.

3.3 Peripheral blood samples

When possible, about 100 ml of PB was taken from CRC patients, who underwent surgery for the removal of tumor, using sterile violet vacutainer tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). After a blood 1:2 dilution in PBS (Euroclone), PBMCs were isolated by Ficoll-Paque^{TM} PLUS (GE Healthcare) density gradient centrifugation. Differential migration during centrifugation results in the separation of cell types into different layers (Figure 5).

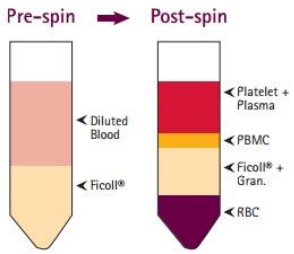


Fig.5: Sample layering before and after Ficoll density gradient centrifugation.

The bottom layer contains Ficoll-aggregated red blood cells (RBC). Immediately above, there is a diffuse layer containing mostly granulocytes and unbound Ficoll. Due to a slightly lower density, the lymphocytes (including the monocytic PBMC fraction) sediment at the interface between the Ficoll and uppermost plasma/platelet layer. PBMCs are removed from the interface and subjected to multiple washes in PBS to remove any residual Ficoll. Isolated cells were stained with 0,4% Trypan blue

(Euroclone), and counted in a Burker chamber under a light microscope. On the basis of cellular count, 4-5 aliquotes of PBMCs were cryopreserved in freezing medium for the setup of future experiments, while PBMCs who remained were used immediately for the subsequent manipulations.

3.3.1 Cells isolation through magnetic labeling

Cells labelling with magnetic beads is one of the most recent approaches used to isolate the cellsm of interest starting from a set of cell populations. As reported by Miltenyi Biotech, MACS® Separation Columns (MS) were developed for the fast separation of any cell type labeled with MicroBeads, human (Miltenyi Biotech). The cells obtained, ready to use for experimental design, showed excellent yields and purities, preserving their functionality. The matrix of the MS columns is composed of ferromagnetic spheres, which are covered with a cell-friendly coating allowing fast and gentle separation of cells. When placed in the magnetic field of a MiniMACS^{TM} Separator (Miltenyi Biotech), the spheres amplify the magnetic field by 10,000-fold, thus inducing a high gradient within the column. This is crucial for isolation of cells which are only minimally labeled with MicroBeads, human (Miltenyi Biotech), leaving enough epitopes free for concurrent antibody staining. The space between the spheres is several times larger than primary and most cultured cells. This allows the cells to freely flow through the column. Magnetically labeled cells are held in suspension within the column and do not actually “bind” the column matrix. This suspension minimizes stress on the cells and allows for efficient sterile washing by avoiding cell aggregation. This methodology was choose to obtain an efficient and high purity isolation of three cell populations: $CD8^+$ T-cells, $CD14^+$ monocytes, and $CD4^+$ T-cells. All those cell populations were isolated by a positive immunomagnetic selection.

3.3.1.1. T CD8+ lymphocytes isolation

$CD8^+$ T-lymphocytes were isolated from PBMC *via* positive immunomagnetic selection. PBMC were centrifugated at 300 x g for 10 minutes; then, supernatant was completely removed. Cell pellet was resuspended in sterile, cold, AutoMACS^{TM} Running Buffer (MACS buffer, Miltenyi Biotech). Specifically, cells were resuspended in 80 µL of MACS buffer with the addition of 20 µL $CD8^+$ microbeads (Miltenyi Biotech) per 10^7 total PBMCs; cells were mixed well and incubated on ice for 15 minutes. Cells were then washed using 1-2 mL of MACS buffer and resuspended in 500 µL of the same buffer. The labelled preparation was passed through a MS separation columns (Miltenyi Biotech) attached to magnetic field (MACS MULTI STAND, Miltenyi Biotech) and the flow-through cells recovered in a falcon as negative fraction and used for subsequent T-cell magnetic labelling. Loading columns were washed three times using 500 µL of MACS buffer following the manufacture's instructions. To elute the fraction containing the magnetically labelled cells, 1mL of MACS buffer was added after the column was removed from the magnetic field and immediately flushed out using a sterile plunger (Miltenyi Biotech). The positive cell fraction was stained with 0,4% Trypan blue (Euroclone), counted in a Burker chamber under a light microscope, and evaluated by flow cytometry. The isolated T $CD8^+$ cells were than cryopreserved for the subsequent experiments.

3.3.1.2. T $CD4^+$ lymphocytes isolation

The T $CD4^+$ cells isolation started from the negative fraction of the cell type previously isolated. The negative fraction was centrifuged, and the cell pellet was resuspended in MACS buffer as reported above. The isolation protocol was the same for all the cell population; the only specific reagent for each separation is represented by magnetic micro-beads ($CD4^+$

Microbeads, human, Miltenyi Biotech), that, in this case, are able to bind only T $CD4^+$ cells. Also this cell fraction was stained with 0,4% Trypan blue (Euroclone), counted in a Burker chamber under a light microscope, and evaluated by flow cytometry. The isolated T $CD4^+$ cells were than cryopreserved for the subsequent experiments.

3.3.1.3. $CD14^+$ monocytes isolation

The negative fraction recovered from T $CD4^+$ cells isolation was centrifuged in order to proceed with the $CD14^+$ microbeads ($CD14^+$ Microbeads, human, Miltenyi Biotech) labelling for monocytes isolation. The isolated $CD14^+$ cells were immediately cultured to obtain immature DCs.

3.4 Generation of immature Dendritic cells

$CD14^+$ monocytes were counted and plated in 6 wells at concentration of 600.000 cells/mL in a final volume of 2,5 mL, ready to became, under the appropriate stimuli, iDCs. Before starting stimulation, $CD14^+$ cells were allowed to adhere in RPMI 1640 (Euroclone) medium containing 3% of FBS (Euroclone) from one to two hours at 37°C, 5% CO_2 humidity. After this incubation, the non-adherent cell populations were throw away with PBS (Euroclone) washings. The adherent monocytes were cultured in RPMI 1640 (Euroclone) with 10% FBS (Euroclone), in presence of recombinant human Interleukin 4 (rh IL-4) (500U/mL) (Immuno Tools) and recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (rh GM-CSF) (800-1000U/mL) (Immuno Tools). The day following, the cells were checked at light microscopy to eventually identify some contaminant lymphocytes. If a contamination of lymphocytes was observed in monocyte-DC culture, the medium must be centrifuge, the pellet discarded and the medium with cytokines, added again to the culture. After 2/3 days, half culture medium was removed and replaced with fresh medium containing

the same starting amount of cytokines. At day 6 or 7 of culture, iDCs were counted under a light microscope and subjected to flow cytometry.

3.5 Ultraviolet B (UV-B) irradiation

During the course of experiments it was necessary to perform a UV-B irradiation of different cell populations. The cells irradiation is a methodology to drive the cell population of interest in a process of programmed cell death, know as apoptosis. The apoptosis of several cell populations (primary tumor cell line, T $CD4^+$ lymphocytes and autologous PBMCs) was evaluated by flow cytometry, using Annexin V and Propidium ioduro (PI). One of the earliest features of apoptosis is a morphological change in the cell plasma membrane. This involves the translocation of the membrane phospholipid phosphatidylserine (PS) from the internal layer to the external layer of the cell membrane. In the presence of calcium ions, Annexin V has a high specificity and affinity for PS. Thus, the binding of Annexin V to cells with exposed PS provides a very sensitive method for detecting cellular apoptosis. The vital fluorescent dye PI is used to distinguish between apoptotic and necrotic cells. PI can only enter necrotic cells across a damaged plasma membrane. So healthy cells are negative for both Annexin V and PI, early apoptotic cells are Annexin V positive but PI negative, later apoptotic cells are positive for both, and necrotic cells are PI positive completely.

3.5.1. Primary tumor cell lines UV-B irradiation

Induction of apoptosis in primary colon cancer cell lines was performed in order to obtain a source of tumor antigens, but without the definition of a specific tumor antigen, that can be used to set up and *in vitro* stimulation of T $CD8^+$ lymphocytes. During the apoptotic process, bubble shaped balls called blebs appear on the surface of the tumor cell. The cell then breaks

down into smaller fragments called apoptotic bodies. These fragments are enclosed in membranes so as not to harm near-by cells. These apoptotic bodies are more likely to be captured by DCs that engulf and process them, exposing their peptides in association with MHC I molecules, to T $CD8^+$ lymphocytes, without causing an inflammatory reaction.

Tumor cells (0.2-0.5x10^6 cell/mL) were placed in 6-well plate and subjected to a 200 Grays UV-B irradiation. Before irradiation, medium was removed and retained and tumor cells were covered with PBS. After irradiation, the original medium (RPMI 1% FBS) was added back to the tumor cells and incubated at 37°C, 5% CO_2. Early and late apoptosis of tumor cells were determined 24-48h after irradiation using Annexin V and PI (Becton Dickinson), according to the manufacturer's protocol. Negative controls were performed on not irradiated tumor cells.

3.5.2. PBMCs and $CD4^+$ T-cells UV-B irradiation

A weak UV-B irradiation of PBMCs and T $CD4^+$ cells was also necessary to achieve the aims of this research project.

Autologous irradiated PBMCs play the role of feeders for effector cells and procure them other sources of APCs.

T $CD4^+$ cells are subjected to weak UV-B irradiation that causes the beginning of the programmed apoptosis path; in this way the T $CD4^+$ proliferation is avoided, but keeping on their culture support.

About 3-4x10^6 cell/mL of PBMCs and the same amount of T $CD4^+$ cells were plated in different 6-well plates and subjected to a 30 Grays UV-B irradiation, as the previously described protocol, but in this case, the apoptosis of these cells was evaluated by flow cytometry after 24h of culture.

3.6 Co-culture set up

After the obtaining of primary tumor cell lines and their UV-B irradiation, after the isolation from PB of CRC patients of cell populations of interest, and after the UV-B irradiation of PBMCs and T $CD4^+$ cells, the co-culture experiments, based on the stimulation of $CD8^+$ enriched T-cells by antigen-loaded DCs, could be performed.

Anti-tumor CTLs were elicited using about 200.000 patient's DCs as antigen presenting cells, about 500.000 irradiated (200 Grays) autologous apoptotic tumor cells as the source of tumor antigens, about 400.000 irradiated (30 Grays) T $CD4^+$ cells, and about 1.000.000 patient's CD8-enriched lymphocytes as effectors, in 48-well plate, in IMDM (Lonza) with 10% of human plasma (Lonza), supplemented with recombinant human Interleukin-7 (rh IL-7) (10 ng/ml) (Immuno Tools) and human Interleukin-12 (rh IL-12) (10 pg/ml) (Miltenyi Biotec). After one week, co-culture will be recovered and re-stimulated in the presence of about 1.500.000 adherent irradiated (30 Grays) autologous PBMCs, apoptotic tumor cells and low rh IL-2 (10 U/ml) (Euroclone) dose, in 24-well plate. The same protocol was reiterated for a total of 4 to 5 stimulation cycles, using increasing amount of rh IL-2 (up to 100U/mL) (Figure 6). After every stimulation, the percentage of T-$CD8^+$ cells was evaluated by flow cytometry. This experimental design was similar to the one described by Montagna and colleagues [Montagna et al., 2001)

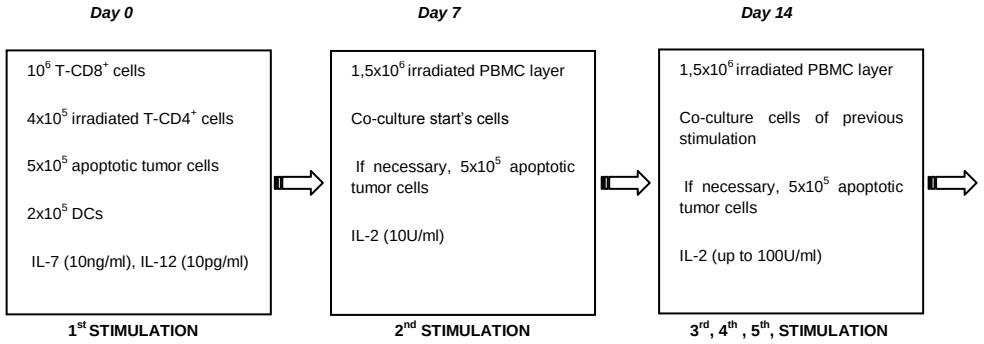


Fig.6: Co-culture planning

3.6.1 Micro-culture set up

In some cases, the different cell populations obtained were not sufficient to set up the co-culture experiment. The primary tumor cell lines, and the T $CD8^+$ lymphocytes and the DCs (when the PB samples are inadequate or of poor quality) are the more critical cell types for our experiments. When one or more of these cell populations were less than expected, a micro-culture was designed in 96-well plate, keeping the same cell ratio used in the co-culture experiment.

3.7 Monoclonal Antibodies and Flow Cytometry

Magnetically isolated lymphocytes from PB of CRC patients, and DCs obtained after monocytes stimulation with rh IL-4 and rh GM-CSF, were assessed by flow cytometry for their specific surface markers.

Primary tumor cell lines were evaluated by anti-EPCAM antibody.

After each co/micro-culture stimulation, the $CD8^+$ lymphocytes and their viability were used for immune monitoring.

Briefly, a small number of these cell populations ($1x10^5$) were stained in FACS Buffer (PBS plus 1% FBS) for 30 minutes at room temperature at dark with respective antibodies.

Surfce marker analysis of *in vitro* cultured cells was performed using a FACScalibur (Becton Dickinson) and the CellQuest (Becton Dickinson) software. We used the following mAbs (all from Becton Dickinson) conjugated to: 1) fluorescein isothiocyanate (FITC): anti-CD3, anti-CD14, anti-CD83; 2) phycoerythrin (PE): anti-CD8, anti-CD1a, anti-CD80; 3) PE-Cyanine5 (PeCy5) or Peridinin Chlorophyll Protein Complex (PerCp): anti-CD11c, anti-CD86, 4) Allophycocyanin (APC): anti-EpCAM, anti-HLA-DR, anti-CD4.

Particularly, lymphocyte populations were analysed using anti-CD45, anti-CD3, anti-CD8 and anti-CD4 mAbs.

Monocytes-DCs (day 6 or 7) were phenotyped using anti-CD14, anti-CD1a, anti-CD11c, anti-HLA-DR mAbs; DCs maturation was evaluated using anti-CD80, anti-CD83 and anti-CD86 mAbs.

7-aminoactinomycin D (7-AAD) (Becton Dickinson) was used to assess DCs viability, and that of $CD8^+$ lymphocytes after each stimulation.

Isotype control antibodies of irrelevant specificities were included as negative controls to detect non-specific antibodies binding.

The percentage of apoptotic primary tumor cell lines, of PBMCs and $CD4^+$ lymphocytes subjected to UV-B irradiation was evaluated using specific 1X Annexin Binding Buffer, Annexin V FITC-conjugated and PI (Becton Dickinson), according to the manufacturer's protocol.

3.8 ELISPOT assay

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies. This method has since be adapted for the detection of individual cells secreting specific cytokines or other antigens. With detection levels as low as one cell in 100.000, the ELISpot is one of the most sensitive cellular assays available. ELISpot assays employ the quantitative sandwich

enzyme-linked immunoabsorbent assay (ELISA): depending on the cytokine/factor analysed, it is between 20 and 200 times more sensitive than a conventional ELISA. In fact, the ELISpot displays similar sensitivity to RT-PCR analysis, but detects secreted protein instead of mRNA, and this is a great advantages because often much cytokines are translationally regulated.

Appropriately stimulated cells are pipetted into monoclonal antibody (specific for a cytokine) pre-coated 96 wells and the micro-plate is placed into a humidified 37°C CO_2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted cytokine. After washing away cells and any unbound substances, a biotinylated polyclonal antibody specific for the cytokine is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. Blue-black coloured precipitate forms at the sites of cytokine localization and appears as spots, with each individual spot representing an individual cytokine-secreting cell. The spots can be counted with automated ELISpot reader systems or manually, using a stereomicroscope.

3.8.1 Twenty-four hours IFN-γ ELISPOT assay

The cytokine ELISpot assay has been widely applied to investigate specific immune responses in cancer.

IFN-γ ELISpot assay (ELISpot ^{PLUS} for Human IFN-γ, MABTECH) is used to monitor CTLs activation against autologous tumor cells, following the manufacturer's instruction. INF-γ ELISPOT analysis were performed,at the end of the third, fourth and fifth co-culture stimulation, and, when possible, at the end of second and third micro-culture stimulation.

Particularly, 96 micro-plates, bought already pre-coated with a monoclonal antibody specific for IFN-γ, were washed four times with 200 µL per well of PBS (Euroclone) and blocked with 200 µL per well of RPMI 1640 (Euroclone) containing 10% FBS (Euroclone) for at least 30 min at 37°C. After incubation, medium was removed and the cells of co-culture, considered as anti-tumor effectors, were added to the plate.

The anti-tumor effectors were plated in triplicate for 24h alone, with autologous irradiated primary tumor cell line, as specific stimulus, o with an aspecific stimulus as positive controls.

Indeed, when measuring antigen-specific T-cell responses in ELISpot assay, the presence of positive/negative controls is a crucial step. In our assay negative control consists of effector cells in medium without specific stimulus, while polyclonal T-cell activator (an antibody specific for CD3 like OK3T, GENE TEX) serves as a positive control both for cell viability and the functionality of the immunoassay. The plate schemes, with the respective cell amounts used in ELISpot assay after a co-colture or a micro-culture, are shown in figure 7:

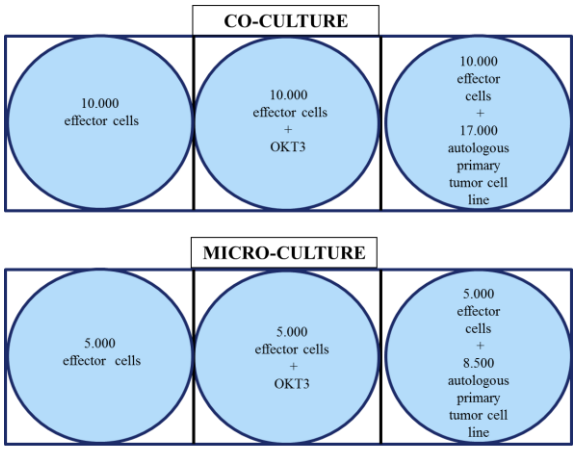


Fig.7: ELISpot Plate representation: each well was performed in triplicate

The plates thus prepared were put in a 37°C humidified incubator with 5% CO_2 and incubated for 24 hours.

The spots detection was done according to manufacturer's instructions.

3.9 Microbiological analysis of primary colon tumor cells contaminations

It is already known that the massive intestinal bacterial flora adversely affects the obtaining of colon primary tumor cell line [Antonic et al, 2013]. Microbiological analysis were conducted to clarify whether the contaminant microorganisms of colon cultures are actually those that reside in this anatomic region or not.

3.9.1 Infectious agents growth on solid medium

About 100µL of 12 tumor cell lines with clear microbiological contaminations were seeded on different solid medium to identificate which infectious agent/s caused the contamination of colon cultures.

Five solid media have been used for this screening: Muller Hinton (MH) (Becton Dickinson), Mannitol Salt Agar (MSA) (Becton Dickinson), McConkey (MC) (Becton Dickinson), Tryptic Soy Agar (TSA) (OXOID) supplemented with serum and Sabouraud medium (OXOID).

MH is a non selective, non-differential medium. This means that almost all microorganisms plated on here will grow.

MSA is a selective medium, because encourages the growth of a group of certain bacteria while inhibiting the growth of others; indeed, it contains a high concentration of salt, making it selective for gram positive bacterium *Staphylococci*. It is also a differential medium for mannitol fermentors, since it contains mannitol and phenol red as pH indicator.

MC is another selective and differential medium, encouraging the growth of Gram negative bacteria, and characterizing those able to ferment lactose to those that not do that.
TSA plus serum is a common medium enriched of human serum to encourages the growth also of most demanding microorganisms.
Sabouraud agar medium is a complex medium useful to observe fungi growth.

3.9.2 Gram staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on their differenT-cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells pink or violet.
Gram staining involves three processes: staining with a water-soluble dye called crystal violet (Sigma-Aldrich), decolorization, and counterstaining, with basic fuchsin (Sigma-Aldrich). Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the basic fuchsin in the final staining process.
Particularly, a colon cell line sample was put on a slide to be stained: the slide was carefully passed on a Bunsen burner three times to heat fix the sample; the crystal violet was added to the sample/slide for 1'; after a water washing, a Lugol solution (Sigma-Aldrich), that is a mordent able to fix the crystal violet to the bacterial wall, was addes for 1'; sample/slice was rinsed with acetone for 3'' (if the alcohol remains on the sample for too long, it may also decolorize Gram positive cells) and after that, with a gentle stream of water; the basic fuchsin was added to the slide and incubated for 1'; a wash

with a a gentle stream of water for a maximum of 5 seconds completed the protocol staining. If the bacteria is Gram positive, it will retain the primary stain and not take the secondary stain, causing it to look violet/purple under a microscope. If the bacteria is Gram negative, it will lose the primary stain and take the secondary stain, causing it to appear pink when viewed under a microscope.

3.9.3 Catalase and oxidase tests

In order to differentiate an infectious agent from others, the specific enzymatic profile of microorganisms could be used.

Catalase is an enzyme possessed by some microbial groups. This enzyme breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Catalase test (Sigma-Aldrich) is useful to differentiate *Staphylococcus* from *Streptococcus* species; it is performed on a slide where a colony is diluted in a drop of H_2O_2. Catalase producing bacteria (catalase positive) that are usually aerobic or facultative anaerobes, release oxygen which forms bubbles.

Cytochrome c oxidase is a component of the cytochrome oxidase system in electron transport chain. This enzyme is present in particular microbial groups, and it is able to oxidize aromatic amines-with production of colored compounds. Oxidase test (Sigma-Aldrich) is usually used to discriminate *Micrococcus* from *Staphilococcus* species, in particular to identificate *Neisseria* and *Pseudomonas* species. The test is performed by dropping a well-isolated colony on solid medium, or on loopful of bacteria placed on the filter paper containing reactive amine-aromatic: the appearance of a purple color indicates a positive reaction.

All bacteria positive for catalase and negative for oxidase tests were subjected to an enterotube test (Becton Dickinson) for *Enterobacteriaceae* members identification.

3.9.4 Enterotube test

Enterotube is a multiple-test system which combines multiple biochemical tests useful in identification of members of the family *Enterobacteriaceae.* Titsworth and colleagues described the enterotube procedure and the results interpretation in 1969 [Titsworth et al., 1969] and this procedure is still in use.

Enterotube is a self-contained, compartmented plastic tube containing 12 different media that allow the determination of 15 biochemical reactions (glucose, gas production from glucose, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide (H_2S), indole, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer (VP), dulcitol, phenylalanine deaminase (PA), urea and citrate). The enclosed inoculating wire allows inoculation of all compartments in one step from one or a few single colonies of an isolate. The resulting combination of reactions, together with the Interpretation Guide (codebook), allow identification of clinically significant *Enterobacteriaceae*. We used Enterotube tests of Becton Dickinson.

3.9.5 Minimal inhibitory concentration

The minimal inhibitory concentration is defined as the lowest concentration of antibiotic or antimicotic that inhibits bacterial or fungi growth respectively. MIC data consists of growth or inhibition of growth at each concentration tested after 24 or 48 hours of incubation at 37°C, starting from an inoculum size of 10^5 colony-forming units (CFU)/ml (grown over night in RPMI 20% FBS).

Usually a stepwise two-fold increase of the antibiotic or antimycotic concentrations is used. The antibiotic and antimycotic with respective range of concentration tested were shown in Table 2:

Drugs	Highest concentration	Lowest concentration
Penicillin/streptomycin*	4X	0,12X
Ampicillin*	400 µg/mL	12,5 µg/mL
Kanamycin*	400 µg/mL	12,5 µg/mL
Chloramphenicol*	20 µg/mL	0,6 µg/mL
Tetraciclin*	20 µg/mL	0,6 µg/mL
Gentamicin*	800 µg/mL	25 µg/mL
Amphotericin B*	1 µg/mL	0,03 µg/mL
Fluconazol**	2 µg/mL	0,07 µg/mL
Voriconazole**	32 ng/mL	0,7 ng/mL

Table 2: Concentration range of antibiotics and antimycotics tested in MIC analysis

** (Euroclone) **(Sigma-Aldrich)*

4. RESULTS

Results regarding the establishment of primary colon tumor cell lines, the setting up of co/micro-cultures with respective immunological analysis, both cytofluorimetric and enzymatic, are described in the section Results I.
Results regarding the study of bacterial or fungi colon cell contaminations are described in section Results II.

4.1 Results I

4.1.1 Case Study

Starting in 2010, a total number of seventy-eight colorectal cancer patients were enrolled in the study. Colorectal tumor tissues were collected during surgery in collaboration with San Giuseppe Hospital and Città Studi Clinical Institute, Milan. Tumor biopsies, collected from each patient, were analysed by pathologists to determine TNM stage and transferred to the laboratory as soon as possible, together with a speciemen of PB.
PB samples were obtained from 65/78 (83,3%) patients and 30/65 (46,1%) were not in sufficient amount for performing the experiments.
The study population's features were summarized in Materials and Methods section.

4.1.2 Rationale of the study

ACT immunotherapy involves the reinfusion of immune cells with antitumor activity, obtained *ex vivo*, into cancer patients [Rosenberg et al, 2011; Pedrazzoli et al, 2011]. This experimental approach was successful applied in several kinds of hematopoietic malignancies and solid tumors.
Since the results of new therapeutic strategies in CRC were poor and often conflicting, we tried to develop an adoptive immunotherapeutic approach for CRC patients, retrieving the tumor-reactive T-cells from the PBMCs.
The rationale of this study is shown in Figure 8 and is based on:

- Obtainment of primary colon tumor cell lines, used as source of tumor antigens after UV-B irradiation;
- Generation of DCs, able to capture and to present tumor antigens to autologus T lymphocytes, starting from monocytes;
- Activation of T CD8 lymphocytes (CTLs), that could direct their cytotoxic ability against tumor cells secreting IFN-γ.

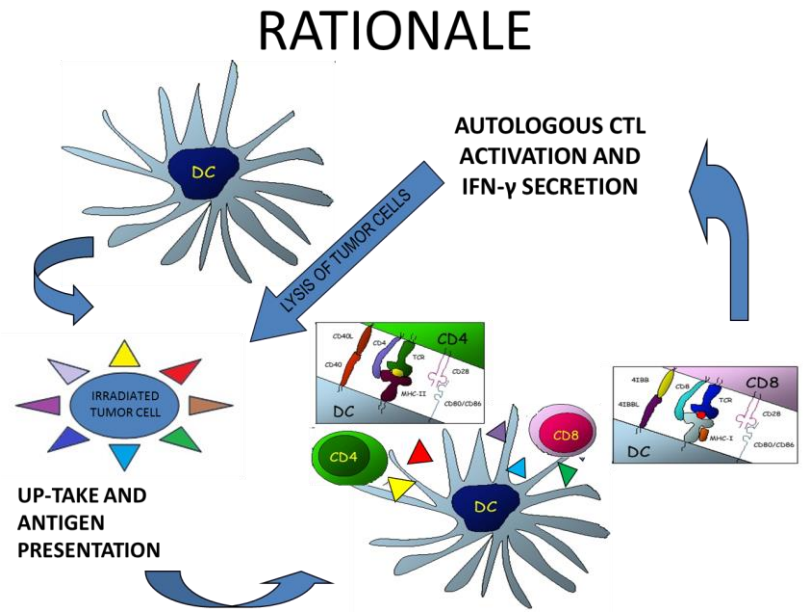


Fig.8: Rationale of our research project

4.1.3 Establishment of primary colon tumor cell lines

Seventy-eight colorectal tumor tissues were collected from 78 CRC patients and were subjected to mechanic dissociation by means of GentleMacs to obtain a single cell suspension. After several steps of filtering and washing, in order to remove all fat residues, cells were cultured in CellGro medium, with the defined antibiotic cocktail as described in Material and Methods section, to allow their adhesion, expansion and to avoid bacterial or fungi contaminations. Nevertheless, 43/78 (55,1%) CRC patients were excluded from the study due to bacterial or fungi contaminations of cell cultures. In addition 15/78 (19,2%) CRC patients were excluded because the tumor cells did not reach a sufficient expansion. Accordingly, the success rate for the generation of primary colon cell lines from fresh tumor tissues was of 25,6%, as primary tumor cell lines were obtained from colon biopsies surgically removed from 20/78 CRC patients. Six of the 20 primary colon tumor cell lines are shown in Figure 9.

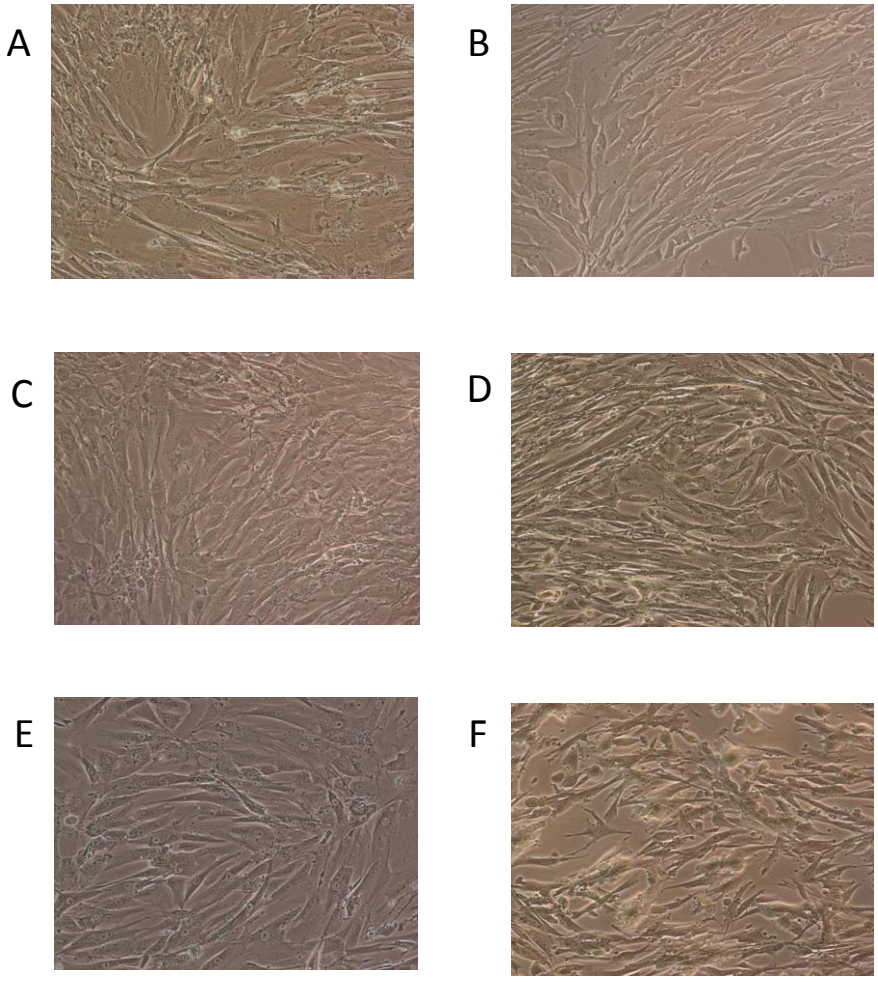


Fig.9: Establishment of primary colon tumor cell lines: cell lines obtained from 6 CRC patients were shown from A to F panels

The phenotypical features of tumor cells were evaluated by flow cytometry using an anti-EPCAM antibody (data not shown).

4.1.3.1 Morphological and phenotypical changes of primary colon tumor cell lines after UV-B irradiation

UV-B irradiation is considered one of the most powerful approach to generate apoptotic bodies from primary tumor cell lines. The apoptotic bodies from primary colon tumor cell lines were generated after a 200 Grays of UV-B irradiation and 48h of culture and were used as source of antigens for T-cells activation by DCs. Morphological changes of cultures, shown in Figure 10, were assessed by light microscopy.

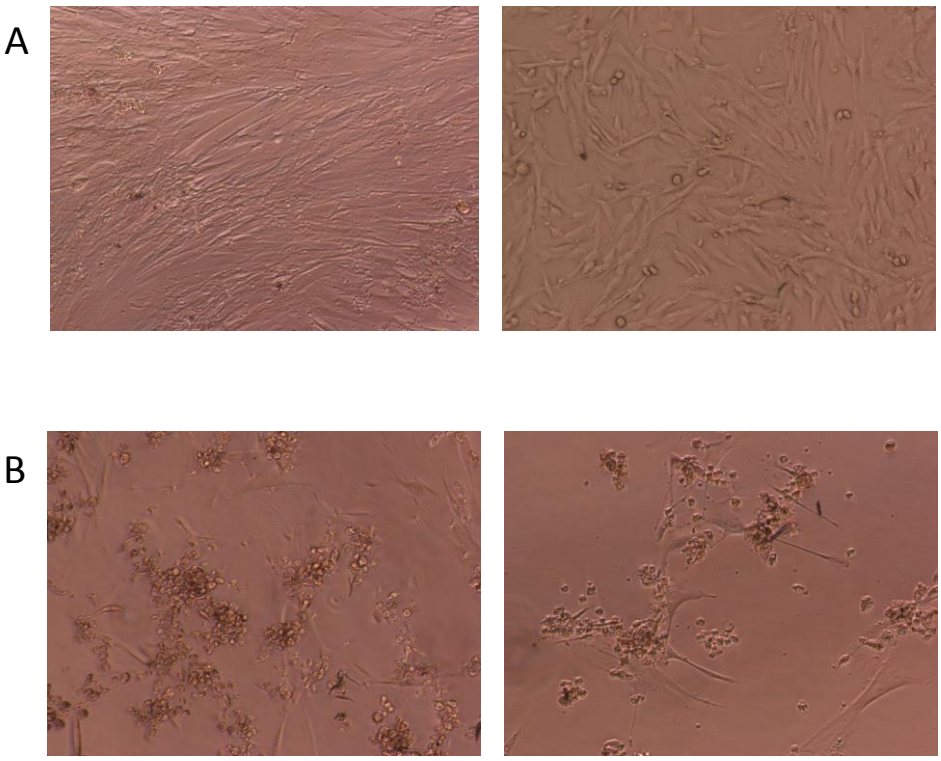


Fig.10: Morphological changes of primary colon tumor cell lines: A) A primary colon tumor cell line before UV-B irradiation; B) A primary colon tumor cell line after 200 Grays UV-B irradiation and 48h of culture

A cytofluorimetric analysis was performed to determine the rate of apoptotic cells obtained after 200 Grays of UV-B irradiation and two days of culture, using Annexin V-FITC and PI staining.

Primary tumor cell lines not subjected to irradiation were used as negative control. Non-irradiated primary colon tumor cell lines contained 2-4% of physiological late apoptotic cells characterized by annexin V^+/PI^+ staining, while after irradiation at 200 Grays and 48h of culture, about 35-40% and 20-25% of early (annexin V^+/PI^-) and late apoptotic cells respectively, were obtained (Figure 11). These percentages were enought to induce the immune stimulation.

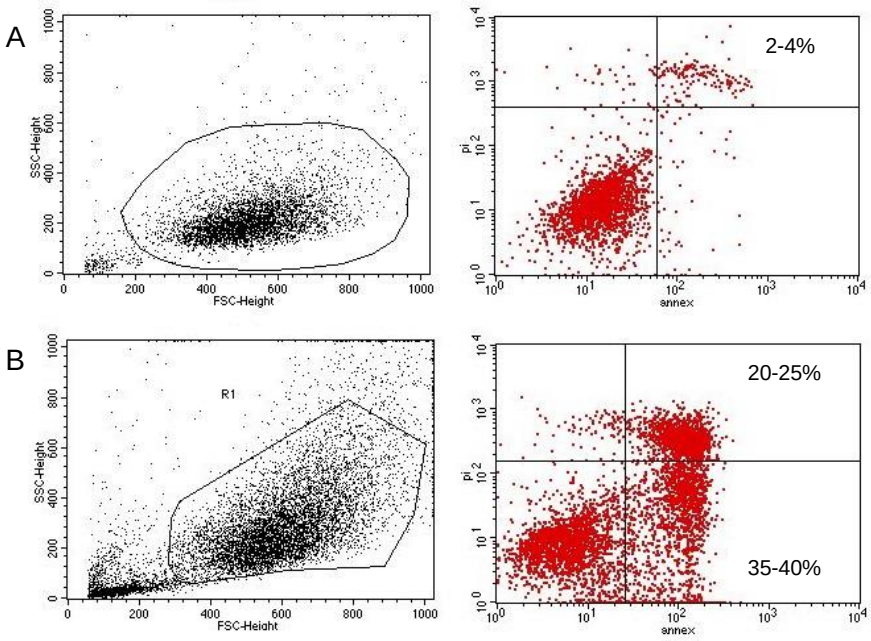


Fig.11:Features of apoptotic tumor cells measured by flow cytometry: UV-B irradiation induced early and late apoptosis in primary colon tumor cell lines. In panel A, the forward/side scatter (FSC, SSC) dot plot, and the dot plot of Annexin and PI staining of a not irradiated tumor cell line were shown. A gate on FSC and SSC dot plot was designed to exclude cell debris. In panel B, dot plots of 200 Gray irradiated tumor cells line after 48 h of culture were shown. A gate on FSC and SSC dot plot was designed to exclude cell debris, and to include cells that, after UV-B irradiation showed an increasing granulosity. Early apoptotic cells were defined by the Annexin V^+/PI^- populations, whereas late apoptotic cells were defined by Annexin V^+/PI^+ populations, and measured by a 2-color flow cytometry analysis. Percentage of annexin V^+/PI^- and annexin V^+/PI^+ cell populations of control and irradiated cell line were shown in the lower right and upper right quadrant of the dot plots respectively.

4.1.4 Morphological and phenotypical features of Dendritic Cells

DCs are the most powerful antigen presenting cells, able to capture, process and present the antigens; therefore DCs play a fundamental role in activating the maincells involved in the anti-tumor immune response: the T $CD8^+$ lymphocytes.

The approach used to obtain DCs starting from magnetically isolated monocytes has been abundantly described in literature.

The advantage in employment of magnetic $CD14^+$ beads lies in obtaining a higher percentage of pure monocytes (>92%) than that usually given by a cell preparation of $CD14^+$ cells (data not shown).

These monocytes were stimulated to differentiate in DCs within 6-7 days of culture in medium containing rh GM-CSF and rh IL-4.

The morphological modifications of monocytes differentiating in iDCs were monitored by light microscopy (Figure 12); ultrastructural characteristics of DCs, such as cytoplasmic projections, abundant organelles and irregular nuclei can be observed in panels B, C and D.

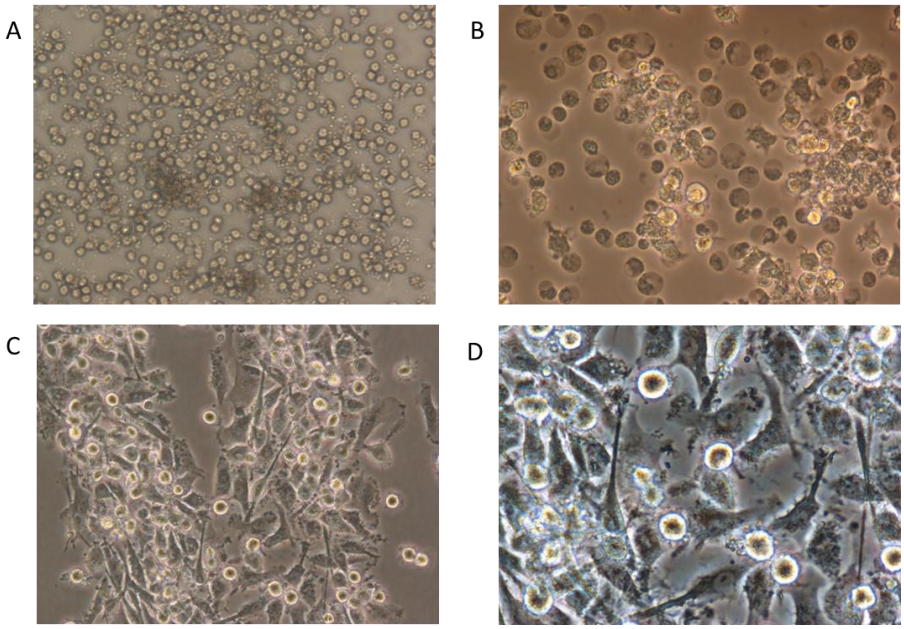


Fig.12: Monocytes differentiation to iDCs monitored by light microscopy: monocytes were cultured in presence of IL-4 and GM-CSF for 6-7 days
A) Monocytes captured at the day 0 of culture; B) Monocytes-iDCs captured at the day 3 of culture; C) and D) iDCs captured at the day 6 of culture

DCs stimulated by these two cytokines are considered to be immature, as reported by flow cytometric analysis.

In order to study the immature DCs population, the following surface markers were analyzed by flow cytometry: $CD14^+$, that is gradually lost during the monocytes-DCs differentiation, and $CD11c^+$, $CD1a^+$ and HLA-DR^+, that are specifically expressed on iDCs. Monocytes differentiation towards a DCs phenotype caused a marked down-regulation of $CD14^+$ positive cells (considering that $CD14^+$ magnetically isolated cells were more than 92%) up to about 10% (data not shown).

The expression of the specific DCs surface markers is shown in Figure 13 A high expression of $CD11c^+$ and HLA-DR^+ surface markers was observed, with about 95% of positive cells for both, while about 35% of $CD1a^+$ cells were reported.

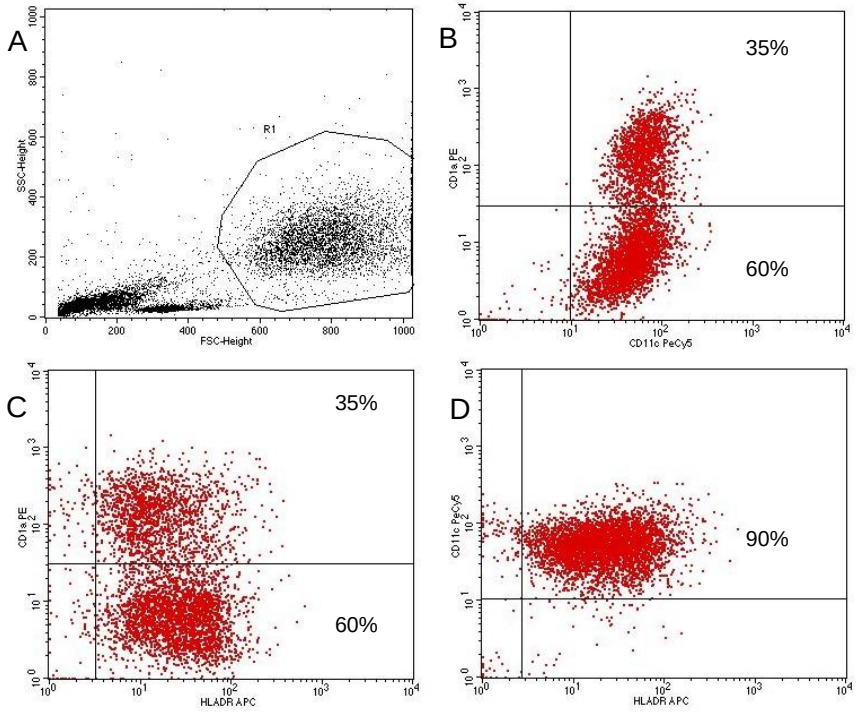


Fig.13: Features of iDCs measured by flow cytometry: dot plot panels showed representative data of monocyte differentiation to iDCs. A gate for DCs analysis was defined in a dot plot of FSC versus SSC (A); leukocyte population was excluded from the gate. CD11c$^+$, CD1a$^+$ and HLA-DR$^+$ surface markers were analyzed. Percentages of cells CD1a$^+$ CD11c$^+$, CD1a$^+$ HLA-DR$^+$ and HLA-DR$^+$ CD11c$^+$ were shown in panels B, C and D respectively.

In addition, iDCs viability has been evaluated using 7-AAD. All DCs surface markers analyzed were negative for 7-AAD, as shown in Figure 14.

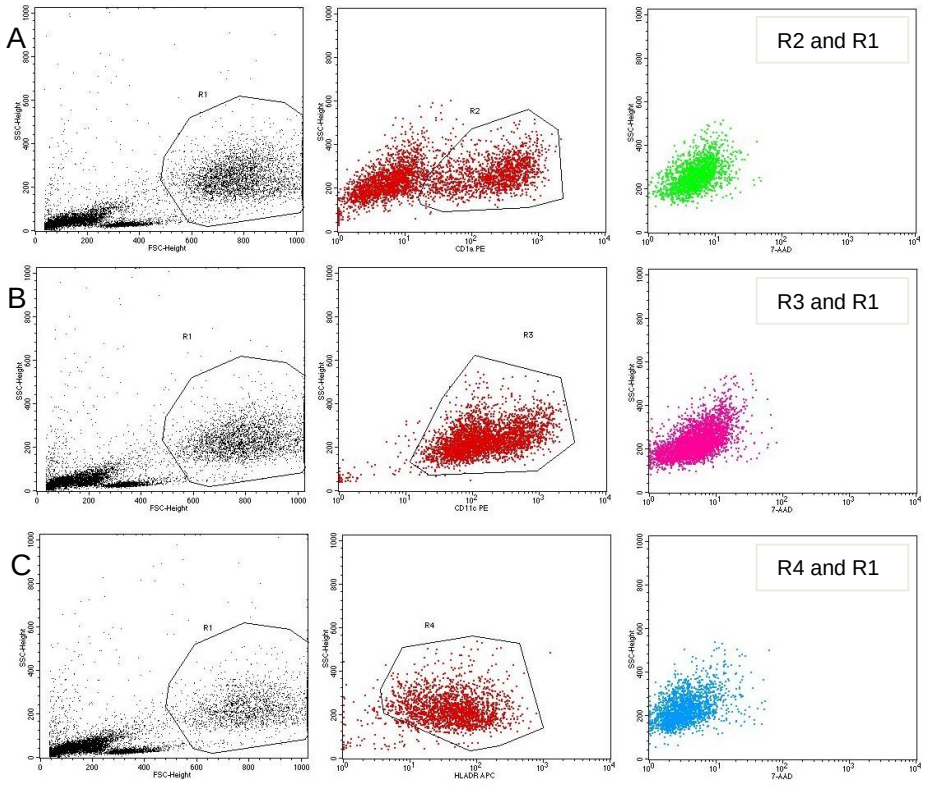


Fig.14: DCs viability measured by flow cytometry. The gate R1 used previously for DCs analysis was kept. The gate R2, R3 and R4 were designed on CD1a$^+$, CD11c$^+$ and HLA-DR$^+$ positive cells and shown in the middle dot plot of panel A, B and C respectively. These gates, subsequently applied to the three SSC versus 7-AAD dot plots, were shown.

It is already known, even if still under debate, that the so generated iDCs, may engulf apoptotic cells and their antigens can be cross-presented for the generation of HLA class I/peptide complexes, allowing the induction of specific CTLs.

4.1.5 Autologous T CD8$^+$ lymphocytes stimulation with dendritic cells pulsed with apoptotic primary colon tumor cell lines in co-culture or micro-culture experiments

On the basis of biological samples collected from CRC patients and of their amount, three co-cultures and three micro-cultures were set up in order to develop a procedure for inducing autologous anti-tumor CTLs.

It was not possible setting up other cultures because 14/20 (70%) of the primary tumor cell lines generated did not have the corresponding DCs.

The cell components of these cultures, the cytokines used as stimuli, and the overall experimental flow chart were illustrated in material and methods section.

A weak UV-B irradiation of T CD4$^+$ lymphocytes and of autologous PBMCs has been performed to retain the ability of these cells to supporting T CD8$^+$ lymphocytes, but without impairing CTLs expansion. An increasing dose of IL-2 was used to allow CTLs expansion.

Furthermore, the culture conditions promoted DCs maturation, that was evaluated by flow cytometry, analyzing some surface costimulatory molecules such as CD80 and CD86, and the surface marker CD83 typical of mature DCs (data not shown).

Five weakly stimulations have been performed for each of the three co-cultures, while three have been done for all the micro-cultures.

These cultures were very difficult to maintain because of their low propensity to expand.

Morphological features of a cell co-culture were monitored by light microscopy at the end of every of the five simulations (Figure 15). DCs, after being co-cultured with immunological effector cells, formed typical non adherent clusters.

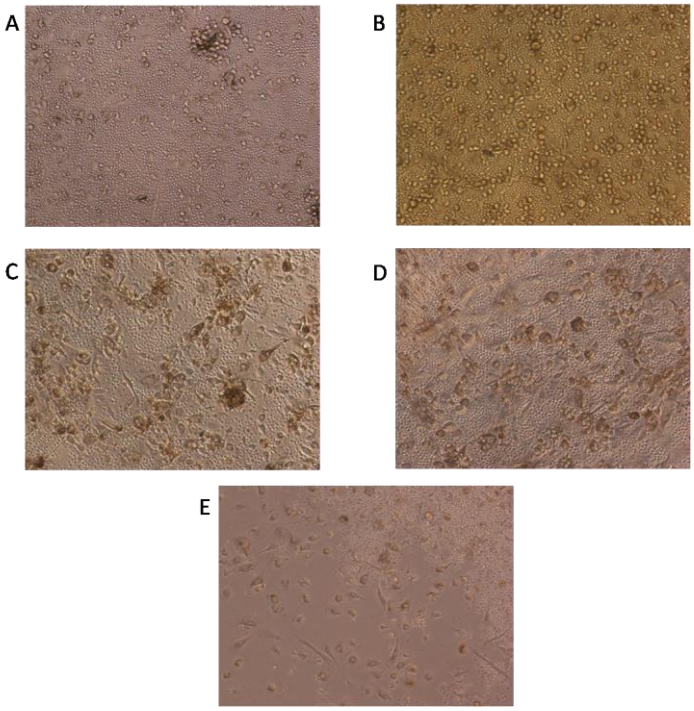


Fig.15: Co-culture monitoring by light microscopy: the morphological features of a CRC patient co-culture were reported at the end of each stimulation (from the 1st to the 5th) from panel A to E

In order to identify the subsets of T-cells reacting to the stimulation with autologous tumor-loaded DCs, followed by restimulation with irradiated tumor cells alone (when necessary), the phenotype of the T-cells from the 6 CRC patients were analized by flow cytometry at the end of every weekly stimulation (data not shown).

The percentage of T $CD8^+$ cells obtained in the co/micro-culture experiments ranged between 7% and 50%. When the percentage of T-cell effectors were >10%, T-cell effectors were cryopreserved for subsequent analysis at the end of each specific stimulation. Particularly, starting from the third stimulation for all the co-cultures and from the second for all the

micro-cultures, about 150,000 and 100,000 effectors cells respectively, were cryopreserved in order to perform an ELISpot analysis.

4.1.6 Evaluation of the anti-tumor immune response with a 24 hours IFN-γ ELISpot assay

The cytotoxic activity of CTLs was defined by a 24 hours IFN-γ ELISpot assay at the end of any specific stimulation, usually from the third to the fifth ones for the co-cultures and from the second to the third ones for the micro-cultures.

The 6 co/micro-cultures were evaluated in response to no stimulus, to aspecific stimulus such as the monoclonal antibody against CD3 (OK3T), or to a specific stimulus with autologous tumor cells, as described in material and methods section.

The example of an ELISpot reader screen, obtained after an ELISpot assay on one CRC patient, was reported in Figure 16; the patient effector cells were analized for IFN-γ secretion at the end of the third co-culture stimulation, as described in material and methods section.

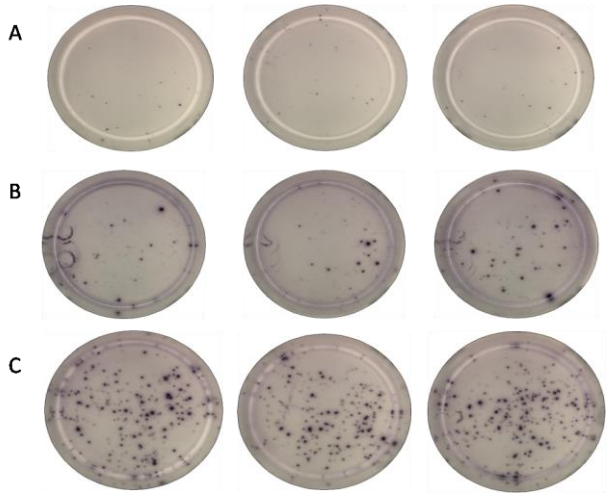


Fig.16: Elispot reader results of a representative IFN-γ ELISpot assay: back-ground IFN-γ secretion of negative controls, represented by the immunological effectors cultured alone, was shown in panel A; the spot forming cells after the immunological effectors culture in presence of an aspecific stimuli, such as the monoclonal antibody OK3T, were reported in panel B; the IFN-γ production of immunological effectors in response to the autologus tumor cells can be appreciated in panel C.

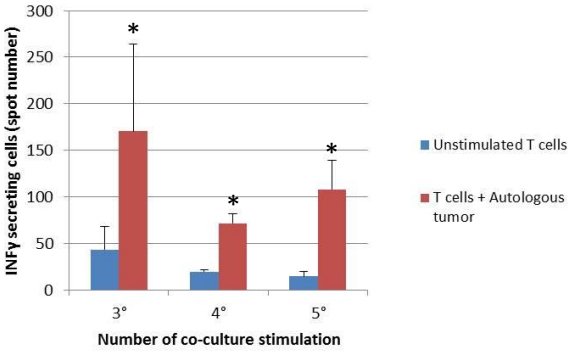
The number of spot forming cells is higher in the wells with autologus tumor cells than in the wells with controls.

An empirical evaluation has been done to determine whether the IFN-γ secretion was significant and at which stimulation, using the following formula:

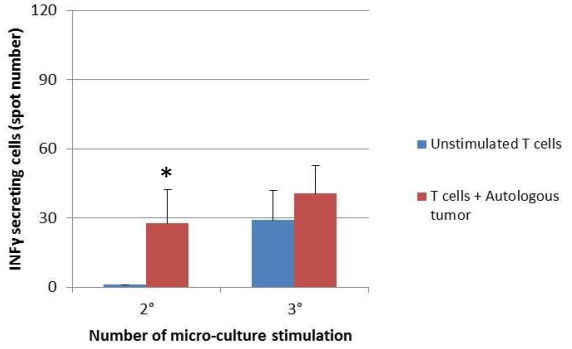
$$[(\text{T-cells} + \text{Autologous tumor}) - (\text{Unstimolated T-cells}) / (\text{Unstimolsted T-cells})] > 2$$

This formula is based on the number of spots observed in the different wells. An histogram representation of all ELISpot results obtained for each patients was shown in Figure 17.

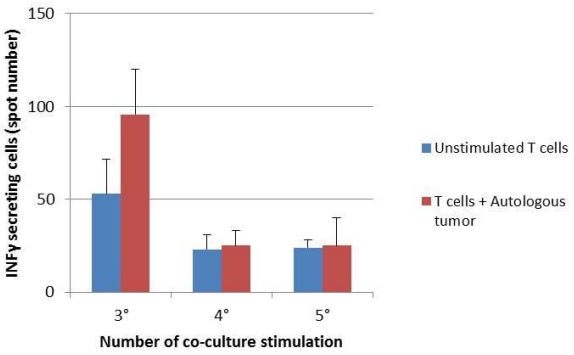
A


300
250
200
150
100
50
0
INFγ secreting cells (spot number)
*
*
*
3°
4°
5°
Number of co-culture stimulation
Unstimulated T cells
T cells + Autologous tumor

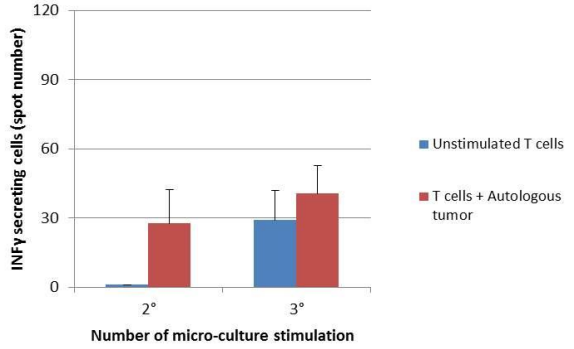
B


120
90
60
30
0
INFγ secreting cells (spot number)
*
2°
3°
Number of micro-culture stimulation
Unstimulated T cells
T cells + Autologous tumor

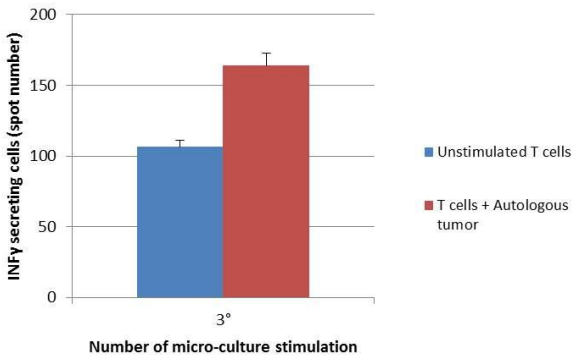
C


150
100
50
0
INFγ secreting cells (spot number)
3°
4°
5°
Number of co-culture stimulation
Unstimulated T cells
T cells + Autologous tumor

D


120
90
60
30
0
INFγ secreting cells (spot number)
2°
3°
Number of micro-culture stimulation
Unstimulated T cells
T cells + Autologous tumor

E



F

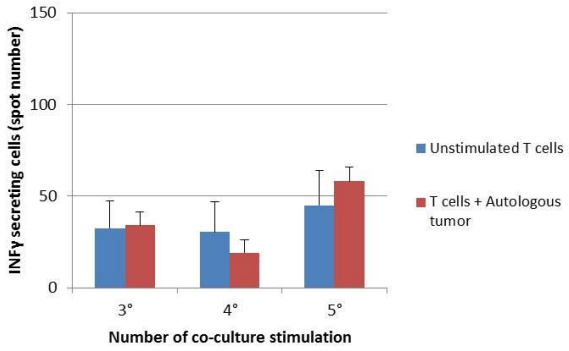


Fig.17: Histogram representation of the ELISpot results:
Six T-cell cultures (from A to F) were evaluated in a 24-hours INF-γ ELISpot assay in response to no stimulus or autologous tumor.
The asterisk indicates a significant secretion of IFN- γ:
** [(T-cells + Autologous tumor) - (Unstimolated T-cells) / (Unstimolsted T-cells)] > 2*

Results of these immunological analysis showed a strong IFN-γ secretion at the end of the third, fourth and fifth co-culture stimulations for one patient, and at the second micro-culture for another patient (Figure 17 A

and B), whereas a weak secretion was detected at third stimulations (one co-culture and two micro-cultures) of three patients (Figure 17 C, D, E).
T-cells from one patient did not react to the stimulation (Figure 17 F).

4.1.7 Results summary

A schematic representation of the obtained results was reported in Figure 18.

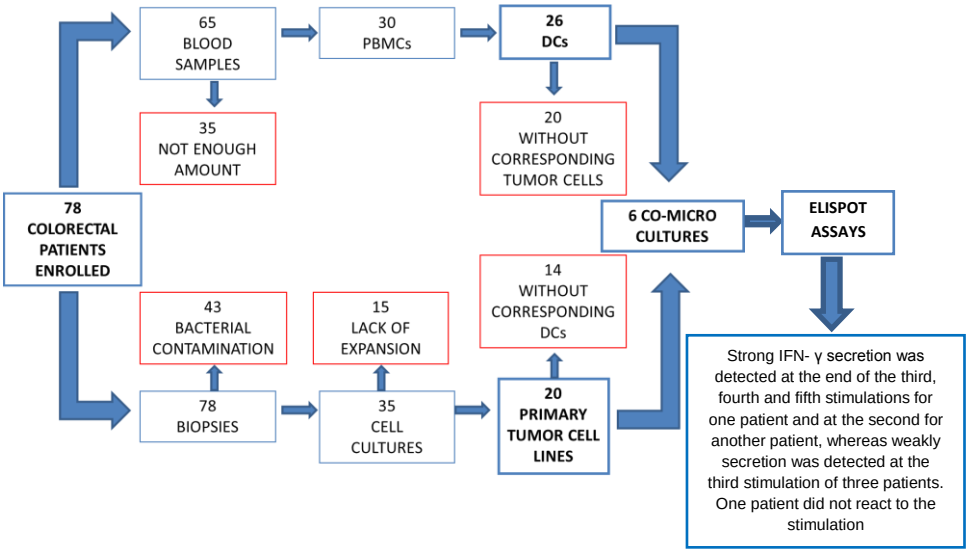


Fig.18: Summary of the obtained results

In this study 78 CRC patients were enrolled. Colon biopsies were collected from each of them, while PB was taken from 65 patients.
Infectious agent contaminations and lack of cell expansion from a side, and poor quality and poor amount of PB samples on the other side, have compromised a larger immunological evaluations. In fact it was possible to setting up only six T-cells co/micro-cultures.

However, a T-cell effectors activation, evaluated by means of ELISpot analysis for the IFN-γ secretion, has been observed. In particular, strong IFN-γ secretion was detected at the end of the third, fourth and fifth stimulations for one patient and at the second for another patient, whereas weakly secretion was detected at the third stimulation for three patients.

4.2 Results II

4.2.1 Infectious agents identification in colon cancer speciemens

Microbiological analysis were performed on 12/43 (28%) colon cell lines, that showed contaminations. In order to verify the presence of infectious agents, 100µL of cell culture were seeded on MH, MSA and MC solid media; in addition 100 µL were seeded on TSA medium enriched of serum to allow the growth of more esigent bacteria in terms of nutrition.

On the same speciemens, a Gram staining was performed. When suspected yeast contaminations were identified by plating staining, the microorganism on Sabouraud agar medium.

In order to proceed on infectious agents identification, catalase and oxidase tests were conducted.

Taken together, the results of these microbiological analysis allowed us to identify that the colon cell lines were often contaminated by more than one microorganism. Among the Gram negative bacteria, *Morganella Morganii* was found in 33% of cases, *Escherichia Coli* and *Pseudomonas* in 22%, while *Serratia* and *Citrobater* in 11%.

Particularly, Gram negative contaminations were found in 42% of colon cultures and 33% of them showed yeast contaminations. Gram positive contamination was found in association with Gram negative bacteria and this type of contamination represented the 8% of the total, while an

association between Gram negative bacteria and yeast was found in 17% of cases (Figure 19).

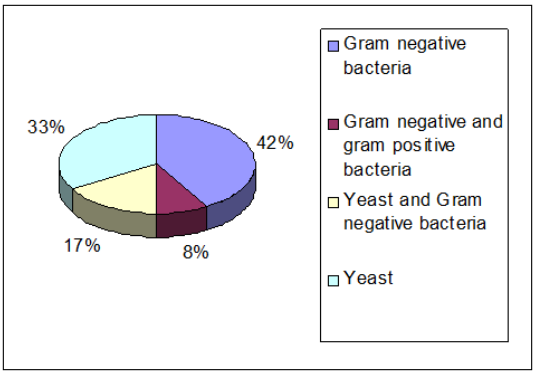


Fig.19: Grafic representation of the colon cell line contaminations

All Gram negative microorganisms, negative at the oxidase test, were subjected to the enterotube test, to identify which member of the *Enterobacteriaceae* family caused the contamination. *Morganella Morgani, Escherichia Coli, Serratia* and *Citrobacter* were the identified Gram negative microorganisms.

Pseudomonas Aeruginosa was the identified Gram negative bacteria, positive at the oxidase test.

The only Gram positive bacterium found in the colon cell line belonged probably to group D *Streptococcus* since: the bacteria grew in chains or pairs; catalase test was negative and the bacteria growth was observed on MH medium.

Yeast contaminations were probably due to *Candida Albicans* (Figure 20).

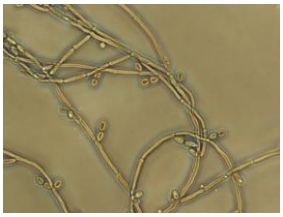
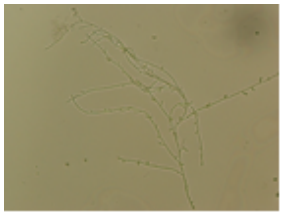



Fig.20: Example of an yeast contamination captured at light microscopy

The types of microorganisms identified as contaminants of primary colon cell lines were summarized in Table 3.

	N°/TOT	TYPE OF M.O.
GRAM (-) Bacteria	9/12	*Morganella M.* *E.Coli* *Pseudomonas* *Serratia* *Citrobacter*
GRAM (+) Bacteria	1/12	*Streptococcus D*
YEAST	6/12	*Suspected Candida*

Table 3: Infectious agents found in the primary colon cell lines

MIC analysis were conducted in order to test the concentration of several types of antibiotics and antimycotics able to inhibit bacterial or fungi growth. We performed this investigation after 24 and 48 hours of incubation of the inoculum of 10^5 CFU/mL, using gentamicin, kanamycin, penicillin/streptomycin, cloramphenicol, and ampicillin as antibiotics and fluconazole, voriconazolee and amphotericin B as antimycotics, using the range of concentrations reported in the Material and Methods section.
Results of these analysis were summarized in table 4 for antibiotics and in table 5 for antimycotics.

M.O.	h	ANTIOBITICS					
		Kanamycin	Ampicillin	Penicillin/ Streptomicin	Gentamicin	Chloramphenicol	Tetraciclin
Morganella M.	24h	S	S	S	S	S	S
	48h	S	R	S *	S ***	R	S ****
Escherichia Coli	24h	S	S ***	S	S	R	R
	48h	S	S ***	S **	S	R	R
Serratia	24h	S	S	S	S	S	S
	48h	R	R	S	S	R	S ****
Citrobacter	24h	S	S ***	S	S	R	R
	48h	S	R	S	S	R	R
Pseudomonas	24h	S ***	R	S *	S	R	R
	48h	R	R	S **	S	R	R
Streptococcus D	24h	R	R	S	R	R	R
	48h	R	R	S **	R	R	R

* until 2x; ** until 4x; *** 400µg/ mL; **** until 20 µg/ mL

Table 4: MIC analysis: infectious agents sensibility (S) or resistance (R) to antibiotics: evaluations after 24h and 48h of incubation

M.O	h	ANTIMYCOTICS		
		Fluconazole	Voriconazole	Amphotericin B
*Yeast**	24h	S	S	S
	48h	S	S Δ	S ΔΔ

* Suspected Candida Albicans; Δ until 0.032ng/mL; ΔΔ until1 µg/mL

Table 5: MIC analysis: infectious agent sensibility (S) or resistance (R) to antimycotics: evaluations after 24h and 48h of incubation

These results showed that many antibiotics were often unable to inhibit the bacteria growth in colon cancer cultures. Gentamicin and kanamycin were the more efficient antibiotics identified by the MIC analysis.

Among antimycotics, only fluconazole was able to inhibit the fungi growth.

5. DISCUSSION

5.1 Immunotherapeutic strategies in cancer

Antitumor immunotherapy for CRC has been studied for decades. Although some clinical trials of cancer immunotherapy have demonstrated a potential benefit for patients with CRC, yet immunotherapy remains only an experimental option for this disease.

Usually, immunotherapy targeting CRC takes one of two approaches: cancer vaccines or ACT.

The development of a cancer vaccine is a complex process that needs the identification of a suitable antigen target and the design of an appropriate vaccine mechanism to elicit immune responses against cancer cells expressing that antigen [Xiang et al., 2013].

The production of several types of vaccines against CRC, including tumor cell vaccines, peptide vaccines, DCs vaccine, DNA vaccine and viral vector-based vaccine, has been more diffucult than vaccine development against other cancers. The main reason is that TSA, TAA and CTA are less expressed in CRC than in other neoplasies [Boncheva et al., 2013].

Anyway, at least ten-tumor associated antigens and thirty-five major MHC restricted epitopes derived from tumor antigens have been identified as potential targets also for T-cell mediated adaptive immune response [Line et al., 2002, Chan et al., 2010].

Although the concept of a preventive vaccine is appealing, current available CRC vaccines are applied to activate the immune system to destroy tumors once they are detectable, and, therefore, are indicated as therapeutic.

As reviewed by Merika and colleagues [Merika et al., 2010], there are several evidences that the immune system is capable of mounting an immune response in CRC, even if it is not always effective in sustaining it or the preventing tumor progression. In fact antigenic stimulation may lead to the generation of a small population of memory T-cells [Sallusto et al.,

2004]; a great number of infiltrating memory T-cells has been associated to decreasing of metastasis [Pages et al., 2005], and intra-tumour lymphocytic infiltration has been shown not only to inhibit tumour growth [Baier 1998] but also to improve overall CRC patients survival [Diederichsen et al., 2003, Naito et al., 1998, Galon et al., 2006].

However, similar to other cancers, many immune evasion mechanisms can be employed by the tumor to escape the immune system responses, even if weak. Among these, the shift from Th1-Th2 immune responses, the loss or down regulation of HLA class I processing and presentation, the defective DCs functions, the T-cell loss of signalling molecules, presence of T regs, TGF-β, VEGF and others have been reported [Evans et al., 2010]. Anyway, many strategies employed to restore these antitumor responses have been studied, and all converged on the necessity to develop vaccines or other immunotherapeutic approaches that trigger CTLs responses, Th1 helper immune responses and to limit the secretion of inhibitory cytokines or the action of inhibitory cell populations.

The main aim of this research project was the development of an ACT approach, potentially useful to introduce a new therapeutic strategy for CRC patients in a near future.

Our study was designed starting from the evidence that ACT was successfully applied in melanoma and RCC patients. Additionally, the best demonstration of the ACT clinical efficacy was reported for EBV-induced lymphoma, using EBV-T specific cell lines and CTLs.

ACT with TIL is an example of a specific, adoptive approach to the therapy that has been proven to be an effective treatment for metastatic melanoma patients. ACT involves the isolation and the identification of antitumour T lymphocytes, from fresh patients biopsy speciemens, and their *ex vivo*

growth, followed by their infusion into the cancer patient [Rosenberg et al., 2009].

Dudley and coworkers reported that among 93 metastatic melanoma patients treated with TIL selected for tumor recognition, an evident response was observed in 50-70% of them, including 15 patients who had complete responses [Dudley et al., 2010].

A comparison of the several published studies on ACT protocols and their clinical efficacy in melanoma patients, such as the adoptive T-cell transfer trials currently recruiting patients, were reviewed by Hershkovitz [Hershkovitz et al., 2010].

There are many reasons for which melanoma has improved the knowledge of cancer immunology, first of all the relative accessibility of melanoma lesions and the fact that melanoma is one of the easiest cancers to adapt to tissue cultures [Maio, 2012], event that allows the identification of TAA, CTA and TSA earlier than in many other tumors with different histotypes [Houghton et al., 2001, Boon et al., 1996].

One of the possibilities for improving ACT for metastatic melanoma and other cancer patients is based on the transfer of genetically modified peripheral T-cells instead of TIL, mainly inducing T-cells to express TCRs or CARs specific for various target tumor antigens.

The frequent toxicity is one of the side effects of these trials. Skin and eyes toxicity has been reported in clinical trials targeting the gp100 and MART1 antigens, even if a melanoma regression was observed [Johnson et al., 2009].

Similar events were reported in RCC, in which carbonic anhydrase IX (CAIX), overexpressed in both tumoral and normal liver, small intestine and gastric mucosa [Leibovich et al., 2007], has been targeted by T-cells expressing a CAIX-specific CAR. An autoimmune cholangitis with no

clinical objective tumor regression was observed [Lamers et al., 2006 and 2013].

During a CRC ACT clinical trial, autoimmune colitis events occurred in all patients treated with T-cells expressing a TCR with high affinity for CEA, that is overexpressed in CRC, but also present in healthy digestive tract.

Additionally, Morgan and colleagues [Morgan et al., 2010] reported severe side effects in CRC patients after the treatment with Her2-specific CAR T-cells.

Taken together, these clinical trial results show that autoimmune toxicity arises when critical normal tissue expresses antigens intentionally or unintentionally targeted by the injected T-cells; this is one of the reasons why the identification of CTA, not expressed in normal adult tissue, could be very important for cancer immunotherapy. In addition, a second type of toxicity, stemed from high cytokine concentration released by the engineering T-cells, could be observed in ACT protocols. However, several approaches to control these phenomenons have been used or are in development, such as the T-cells engeneered to express inducible suicide gene in association with TCR or CAR genes [Di Stasi et al., 2011]. Another strategy is the engeneering of T-cells together with TCR and CAR targeting different tumor antigens, in order to decrease the possibility that those could be simultaneously expressed on normal tissues.

On the basis of the few literature results on the immunotherapeutic approaches in CRC, their safety and efficiency, we have designed the groundwork to develop a new immunotherapeutic strategy in CRC patients, starting from colon biopsy and from the isolation of immune cells from PB, overpassing the isolation of TIL since some studies on this lymphocyte population were already done.

Montagna and colleagues [Montagna et al., 2004] have investigated a procedure to obtain *in vitro* a large amount of anti-tumor HLA restricted CTLs, through the stimulation of patient's $CD8^+$ enriched T-cells, isolated from PB, with DCs pulsed with apoptotic solid tumor as source of tumor antigens. In this way the hard steps of tumor antigens identification and molecular definition are not required.

5.2 Dendritic cells as antigen presenting cells

First of all, to discuss the results obtained in our work it is necessary to underline which procedure has been chosen to generate DCs and the benefits of using apoptotic tumor cells as source of tumor antigen.

The main functions of DCs, their features based on their maturation status, their concentration in PB, their specific surface markers, their use in cancer vaccine strategies and in ACT protocols have been already decribed in the introduction section. Due to DCs low concentration in PB (about 0,01%), several methods have been developed to enriched the DCs population *in vitro*. At the beginning, DCs were obtained starting from the plastic adherence of $CD14^+$ monocytes from PBMC, followed by 5-7 days incubation with rh GM-CSF and rh IL-4 containing medium [Sallusto et al., 1994, Gluckman et al., 1997, Palucka et al., 1998]. Recently, monocytes have been selected by $CD14^+$ positivity, using a magnetic activated cell sorting with magnetic beads, following PBMCs isolation by density centrifugation by Ficol-Paque, in order to obtain a high level of purity of monocytes. Many studies use magnetic labeling to obtain monocytes with high purity [Duddy et al., 2001], as well as other cell populations. The percentage of monocytes purity generated with this method was comprised between 90-98%, as reported from Miltenyi Biotec, and also our results were in accordance with this percentage: about 92% of $CD14^+$ cells were

obtained after PBMCs magnetig labeling with $CD14^+$ microbeads (data not shown).

In a study of Elkord and co-workers [Elkord et al., 2005] the impact of these two different methods, based on plastic adherence or magnetic labeling of monocytes, on DCs functionality has been investigated. The authors showed that the procedure used to isolate monocytes may influence the DCs ability to express costimulatory molecules, maturation markers and to produce immunomodulatory cytokines. In particular, DCs derived from adherence-isolated monocytes secreted higher levels of IL-12, IL-10 and TNF-α than DCs grown from MACS-isolated monocytes. IL-12 has been shown to increase IFN-γ secretion from natural killer (NK) cells and T-cells and the cytolytic ability of these cells, promoting the development of Th1/Tc1 immune responses [Emtage et al., 2003]. However, IL-10 secretion blocks DCs maturation by interfering with the up-regulation of costimulatory molecules and IL-12 production, thereby limiting the ability of DCs to promote Th1/Tc1 responses. The inability of MACS derived DCs to secrete significant amounts of cytokines is probably caused by blocking of CD14 molecules by anti-CD14 microbeads, but the exact mechanism is still unclear [Elkord et al., 2005]. This is the reason why CRC co-cultures need human IL-12 containing medium, while the inhibition of immunosuppressive cytokines secretion is an advantage of the monocytes magnetic isolation methods.

Our results showed that morphological features of DCs obtained from $CD14^+$ magnetic labeling were comparable to those obtained with the same protocols by Daddy and colleagues [Duddy et al., 2001], but also to those obtained by Heo [Heo et al., 2009].

Regarding the cytofluorimetric analysis, employed to investigate the phenotypic features of DCs, we observed that DCs expressed surface

markers of immature DCs and lost $CD14^+$ monocyte surface marker. In particular, CD11c and HLA-DR surface markers expression were comprised between 90-95%, while CD1a between 35-40%. CD14 positive cells were less than 10%. These data were in accordance with others reported in literature [Mucci et al., 2009, Duddy et al., 2001, Von Euw et al., 2007].

The level of DCs maturation was also evaluated in the co-culture, using an antibody against CD83, an immunoglobulin member that has been shown to be up-regulated following DCs maturation [Elkord et al., 2005, Zhou et al., 1996], and antibodies against the costimulatory molecules CD80 and CD86 (data not shown). Indeed, in contrast to studies that performed the DCs maturation after antigen-capture but before the antigenic presentation to T-cells, our co-culture experiments were setting up to allow DCs maturation immediately after the antigen presentation to T-cells.

5.3 Primary colon apoptotic cell lines as source of tumor antigens

In this study, apoptotic CRC primary tumor cell lines have been chosen to deliver tumor antigens to DCs during *ex vivo* manipulations. The principle of this approach is that DCs could present tumor derived epitopes to $CD8^+$ lymphocytes, as others do using different sources of tumor antigen. The effector cells, once activated, may infiltrate the tumor tissue, recognize the tumor antigens and kill the tumor cells, a process largely studied in mouse models [Boissonnas et al., 2007].

Therefore, our approach for preparing and loading tumor antigens on DCs was designed using a whole tumor cells for the induction of multiple antigen-specific policlonal CTLs, in contrast to those that use a single tumor-associated antigen for the induction of a single-specific monoclonal CTL.

Indeed, unlike specific and known tumor associated peptide antigens, whole tumor cells may better apply to many neoplasies for which few or no defined TSA are available, like CRC. In addition, targeting a single TAA or TSA may induce the immune escape of some cell clones which downrugulate the specific antigen. On the other hand, the main disadvantages of this method included a substantial need of tumor material surgically removed and the difficulties of the immunological monitoring in the absence of known target antigens.

In a study by Yasuda and coworkers, the efficacy on anti-tumor immunity of four different strategies for pulsing DCs with whole tumor cell antigens was evaluated in a murine model of colon cancer [Yasuda et al., 2006]. These strategies included DCs pulsed with tumor lysate, freeze-thawed necrotic tumor cells, irradiated apoptotic tumor cells and DC/tumor cell hybrids. Tumor stressed cells, including both apoptotic and necrotic tumor cells, could be captured and processed through MHC class I and II presentation pathways. Therefore, DCs pulsed with these sources of tumor antigens can prime tumor-specific CTLs and Th cells. Despite the fact that the best immunological results of this research group have been achieved with DC/tumor cell hybrids, similar to those reported by Galea-Lauri et al [Galea-Lauri et al., 2002 and 2004] and Kao et al [Kao et al., 2005], it has been shown that irradiated apoptotic tumor cells were able to activate a more powerful anti-tumor response than what freeze-thawed necrotic tumor cells and tumor lysate did in this model.

A critical analysis on the reason why the use of apoptotic tumor cells is better than necrotic cells to induce an anti-tumor immune response was done in a very recent work by Buckwalter [Buckwalter et al., 2013]. The authors also reported the different opinions and results obtained in other studies on wheter apoptotic or necrotic deaths are immunostimulatory

phenomena. They clarified, through *in vitro* and *in vivo* investigations, that apoptotic cells are significantly more immunogenic than necrotic cells, even if the antigenic repertory was the same for the two cell populations. It has been also reported that necrotic cells did not have any immunosuppressive or tolerogenic features, and both could be taken up by DCs in an equivalent manner. An important difference between the use of apoptotic or necrotic tumor cells is that the T $CD8^+$ cells elicited by apoptotic cells expand, accumulate, and express effector functions, while those primed by the necrotic cells do not [Buckwalter et al., 2013], because necrotic cells fail to engage CD40, lacking immunogenicity. These results showed that despite apoptotic death is a physiological process, apoptotic cells are perfectly able to elicit an immune response, trasfering the antigens for cross-presentation to DCs, that are able to present the antigens to T naïve cells. A possible explanation for the absence of autoimmune responses during apoptosis could be found on the fact that the "physiological apoptotic cells" express self components, while non-self components, able to induce a T-cells activation, may be often found in apoptotic tumor cells.

We observed that the optimal percentage of about 35-40% of early apoptotic primary CRC cell lines can be obtained after 200 Grays UV-B irradiation and 48h of culture; this experiment was optimized using different grades of irradiation and time of incubation, such as 24, 48 and 72h, after UV-B irradiation (data not shown). The morphological changes of primary tumor cell lines after the optimized experimental conditions showed the generation of apoptotic bodies, also called blebs, that are more easily captable by DCs, able to present the antigens in association with MHC class I molecules to CTLs.

In this project, the ability of DCs to capture the apoptotic bodies was not evaluated singularly. In contrast with other studies that pulsed DCs with

apoptotic tumor cells or other sources of tumor antigens before starting the T-cell stimulation, we performed simultaneously the antigens-DCs uptaking and the DCs cross priming to the T-cell effectors, given an overall consideration after the co-culture experiments. This procedure was necessary in order to reduce the lost of DCs and tumor cells during the steps culture and may represent an experimental limitation, because we could not verify the efficiency of the antigen up-taking. At the end of the co-culture stimulations, an efficient antigen presentation was supposed to have occurred, if IFN- γ was detected.

5.4 Success rates of colorectal cancer cell lines establishment

The scarse evidence that CRC may induce tumor specific T-cell responses in an autologous setting [Dalerba et al., 2003], contrary to other cancer such as melanomas, could be due to the difficulty in obtaining stable CRC cell lines.

As reported in results section, althogh 78 CRC patients were enrolled in this study, and although colon biopsies were collected during surgery from each of them, just 20/78 (25,6%) CRC cell lines were obtained.

Numerous previously studies have reported the difficulty to initiate cancer cell lines from fresh surgically removed colon tumors [Namba et al., 1983, McBain et al., 1984, Kirkland et al., 1986, Oh et al., 1999]. However our success rate of 25,6% in obtaining primary tumor cell lines from colon biopsies was higher than those reported in other studies cited. In 2007, a very interesting study was conducted by Dangles-Marie and colleagues [Dangles-Marie et al., 2007] in order to increase the success rate of cell line establishment by engrafting tumor fragments in immunocompromized mice before the *in vitro* culture step. Additionally, a comparison between the cell lines obtained directly from tumor speciemens and from the corresponding established xenografts was made. Thirtyone surgical speciemens were

used for the direct *in vitro* establishment protocol: only 3/31(9,7%) speciemens grew immediately in cell culture. Since all the cell lines were generated from primary tumors and none from colon metastasis, the success rate in establishment of CRC lines from fresh primary tumors was comparable to that obtained in our project (25,6%), Otherwise, a better success rate was obtained by the authors after xenotransplantation: briefly, 20 xenografts were established from 26 tumor speciemens (77%) and 47% of cell lines were generated. Half of them was obtained from primary tumors and half of them from tumor metastasis. In addition, they demonstrated that colon cancer cell lines obtained from xenografts grew faster than those generated directly from primary tumor. Conversly, in 2011, Sarrabayrouse and coworkers were able to obtain only one (5,8%) tumor cell line from 17 primary CRC speciemens [Sarrabayrouse et al., 2011].

5.5 Infectious agents' contaminations in colorectal cancer cell lines

The massive intestinal bacterial flora adversly affected the obtaining of colon primary tumor cell lines from fresh colon biopsy; indeed, the main problem was represented by microbiological contaminations of the cultures, despite a large antibiotics and antimycotics spectrum was used.

Penicillin/streptomycin, gentamicin and amphotericin B (fungizone) were added in the transport medium of the biopsies, and also in the medium culture. Neverthless, the percentage of colon cell lines contaminations found in this study was of 43/78 (55,1%).

In a recent review by Antonic and co-workers [Antonic et al., 2013], the current level of evidence that either supports or countradicts that the infectious may contribute to the development of CRC, was summarized and critically assessed. Physiologically, colon is populated by about 10^{14} bacteria; these resident bacteria are fundamental in the development and

function of the mucosal immune system; they help to prevent the pathogenic bacteria colonization and are useful to maintain the physiological microenvironment. It has been hypothesized that the presence and the quantity of bacteria in this anatomic district could favor the development of CRC, with a risk factor 12-fold higher compared to the small intestine and other gastrointestinal tracts, that contains less bacteria. In a study by Tjalsma and colleagues [Tjalsma et al., 2012] a model called "driver-passenger" has been proposed to identify some of the mechanisms by which bacteria could affect normal or susceptible cells, inducing a malignant phenotype. In addition, using a nex generation genomic sequencing approach, they proposed that each stage of CRC development could be associated with a specific pathogen(s) [Tjalsma et al., 2012]. The identification of bacteria involved in early stages of CRC development could be useful to perform both an early tumor diagnosis and to identify subjects with a higher risk to develop CRC.

Recent studies described the role of infectious agents as risk factor in the development of CRC, and also as drivers of tumorigenic process. Thus, we tried to clarify the origins of the cell lines' contaminations, in particular to improve the protocol for the establishment of primary tumor cell line, starting from fresh colon biopies.

As reported in the results II session, the infectious agents found in colon cultures were mainly commensal microorganisms of intestinal tract, able to cause opportunistic infections. Enterotubes allowed us to identify *Morganella Morganii, Escherichia Coli, Citrobacter*, and *Serratia*, whereas by means of other tests, *Pseudomonas*, groups D *Streptococcus* and yeasts have been identified.

Results of MIC analysis underlined that many antibiotics, generally used in cell biology but also extensively used for the therapy, even if tested at very

high concentrations, were unable to inhibit the growth of infectious agents in colon cancer cultures. Gentamicin and kanamicin were the antibiotics at the largest spectrum identified by the MIC analysis. Unfortunately, the gentamicin, that was always added in our cultures, was not sufficient to contrast the infectious microenvironment, probably because of the presence of various types of microorganisms. The same observation should be done about the antimycotics, since only fluconazolo was able to inhibit the fungi growth. However, this is an antimycotic of new generation, and neither its use in cell culture nor the tolerable concentration have been yet established. These results on colon cell culture contaminations are in agreement with the great difficulties previously reported to obtain primary tumor cell lines from colon biopsies.

In a recent work by Wang and co-workers *Bacteroidesfragilis, Enterococcus, Escherichia/Shigella, Klebsiella, Streptococcus and Peptostreptococcus* were found significantly more often in the gut microbiota of CRC patients than in the healthy volunteers [Wang et al., 2012]. Among them, *Klebsiella (Pneumoniae), Streptococcus (bovis), Escherichia Coli,* together with *Helicobacter Pylori* and *Fusobacterium* [Reviewed by Antonic et al., 2013] showed to be more strongly involved in CRC development. In our study, on 12 colon culture investigated, 2/12 (16,6%) were due to *Escherichia Coli*, and 1/12 (8,3%) to a group D Streptococcus, but these data are not sufficient to speculate on a possible role of these agents in CRC development. In addition, the experimental conditions of our microbiological analysis do not permit us to exclude *Helicobacter Pylori* as contaminant agent of the colon cultures. Indeed, the difficulty of growth of *H.Pylori* is very well known, as well as its association with the CRC development, that was largely described in a review by Antonic and co-workers [Antonic et al., 2013]. Finally, the strong presence

of yeasts in our culture, particularly of suspected *Candida Albicans* (6/12, 50%), led us to consider that the chemotherapic regiments of CRC patients could have increased the adhesiveness of yeasts to the gut mucosa.

5.6 Ex vivo T-cells stimulation against autologous tumor

Despite the great difficulties met in obtaining the primary colon cell lines, we have set up 3 co-cultures and 3 micro-cultures in order to activate, in autologous manner, the CTLs against the autologous tumor.

A cytofluorimetric analysis to evaluate the percentage of CTLs obtained at the end of each stimulation of both co-cultures and micro-cultures was done (data not shown). Since T-cells behavior was fluctuating in the co/micro-cultures, we established that T-cell effectors should be subjected to subsequent analysis when their percentage was higher than 10%. However, an increased amount of CTLs was observed up to the end of the third stimulation, while a progressive decreasing was observed after that (with the exception of on patient in which the percentage of CTLs is increased during all stimulations). We hypothesized that this decreasement could be due to a reduction of cell viability, despite the progressive stimulus with increasing doses of rh IL-2, and to a not effective T $CD4^+$ irradiation. Indeed, in some cases, it was possible that Th, that did not successfully completed the apoptotic death process, subtracted cytokines and the nutrients to CTLs, preventing their expansion.

A similar approach was developed by Kurokawa and colleagues in RCC [Kurokawa et al., 2001], one of the most immunogenic known tumor. Both tumor lysate and apoptotic bodies were used as source of antigens for loading DC at 3:1 (tumor:DC) ratio and were kept in culture for 24h; subsequently DCs maturation was performed adding the lipopolisaccaride (LPS) to the culture. Then tumor-specific CTLs were generated co-culturing PBMCs with autologous irradiated tumor cells, and weekly stimulation was

performed , either with antigen-loaded DCs or irradiated tumor cells. The authors showed a temporary descrease of T $CD8^+$ lymphocytes (from 35,3% at the beginning of co-culture, to 5,3% at day 14) and an increase of T $CD4^+$ cells (from 40% to 85%) after 2 weeks of stimulation. Later on they showed a decrease of T CD4 cells (up to 18,6%) and an increase of T $CD8^+$ lymphocytes (up to 79,6%), after 4 weeks and additional restimulations with irradiated autologous tumor cells alone. The authors have analyzed those results underlining that: 1) the use of tumor cells alone as stimulator cells generated a NK cell population; 2) the use of tumor-loaded DCs alone led to induction of $CD4^+$ T helper response after 4 weeks of culture; 3) only the sequential induction protocol of a 2-week starting culture with tumor-loaded DC followed by tumor cells alone was efficient to generate high numbers of tumor specific CTLs [Kurokawa et al., 2001].

On the basis of these results, in our immunotherapeutic study on CRC, effector cells were stimulated with DCs able to load apoptotic tumor cells, and weekly restimulated with tumor cells alone. The presence in co-culture or micro-culture of weakly irradiated T $CD4^+$ lymphocytes should guarantee the immunological support to T $CD8^+$ lymphocytes, without interfering with their specific anti-tumor activation.

An important demonstration of clinical utility of anti-tumor CTLs in adoptive immunotherapeutic strategies, also in solid tumor, was achieved by Turin and co-workes [Turin et al., 2007]. In this study 4 patients affected with solid tumor (1 RCC, 1 ovarian cancer and 2 sarcoma) were enrolled; a large amount of autologous anti-tumor CTLs were produced for each of them, in compliance with Good Manufacturing Practices (GMP), for *in vivo* use.

5.7 Immunoenzimatic evaluation of T-cells activation

The main goal of monitoring antigen-specific T-cell responses in immunotherapy trials is to determine whether treated patients mounted a response following an immune intervention, and whether the detected response is associated with clinical event. In our *ex vivo* study, the definition of the immune response is a prerequisite for hypothesizing their *in vivo* clinical relevance.

IFN-γ is one the main cytokines able to induce opposing effect on tumors, and its role in promoting antitumor immunity has been exensively reviewed by Brassard et al., 2002, Dunn et al., 2006 and Ikeda et al., 2002 and colleagues.

Several IFN-γ detection methods have been developed and are commercially available, all with specific discriminating features. These methods include mainly ELISpot assay, ELISA, and Intracellular Staining (ICS). In the ELISA assay, the cytokine of interest is searched and quantified in the cell culture supernatant using antibodies for the specific capture and detection, and measuring the release of the product of a substrate's color reaction [Cox et al., 2006]. This assay, in contrast to ELISpot, gives no information about individual cells and cannot be used to enumerate the reactive cells [Clay et al., 2001]. Indeed, the main difference between ELISA and ELISpot is that the first one measures the total amount of a cytokine released from all cells in the test, while the ELISpot assay detects the release of the cytokine from a single cell. Various studies supported the higher level of sensitivity of the ELISpot compared to the ELISA assay [Ekerfelt et al., 2002, Mäkitalo et al., 2002, Cox et al., 2006]. Nowadays, the ELISpot assay is used in a wide range of application, including the monitoring of immune responses of cancer patients undergoing immunotherapeutic treatment, the monitoring of the vaccine

responses, and during infectious, neoplastic and autoimmune diseases [Cox et al., 2006]. However, one of the disadvantages of the ELISpot assay is the inability to define the exact phenotype of the cytokine-secreting cells. Consequntly, the ELISpot assay experimental design, that includes opportune controls is foundamental to be sure that the cytokines production is attributed to that specific cellular type. This inconvenience is not present in the ICS method, that followed by cytofluorimetric analysis, so that, both the intracellular trapped cytokines and the surface markers can be detected and the exact phenotype of the cell secreting the cytokine could be determined [Maecker et al., 2005]. Several studies have been designed to investigate the reproducibility of the results obtained using these three cytokines detection methods [Asemissen et al., 2001, Karlsson et al., 2003, Letsch et al., 2003, Sun et al., 2003, Tassignon et al., 2005, Whiteside et al., 2003]. They are substantially comparable and the choise of the method depends by the experimental design and aims. We have chosen the ELISpot assay to evaluate the CTLs IFN-γ secretion at the end of each co/micro-culture stimulation, because of high sensibility and reduced required material, compared to both ELISA and ICS. The choise of positive and negative controls was very important for the validation of results. As negative controls the effector cells were cultured alone, in order to detect the secretion of IFN-γ in response to no stimulus. Positive controls, were represented by the effector cells cultured in presence of an antibody against CD3, called OK3T, that is a polyclonal T-cell activator. The specific stimulus for T-cell effectors was represented by a specific ratio of autologous primary tumor cells.

The results of the ELISpot were analysed using empirical evaluations, frequently reported in literature [Lewis et al., 2000, Janetzki et al., 2005, Cox et al., 2005, Dubey et al., 2007], even if many other statistical methods

have been proposed for the immune response determination [Hudgens et al., 2004, Moodie et al., 2006]. Both statistical and empirical methods have strenghts and limits and they are alternatively used depending on the need of the design. The Student's *t test* [Herr et al., 1998] is the most commonly used, due to the easy calculation of the *p* value. It assumes that the samples size is large enough to assume that the test statistic follows a Student's *t* distribution or that the data are normally distributed [Moodie et al., 2010]. However, ELISpot data do not always satisfy these assumptions. Indeed, this test could not be used in our study, because triplicate wells were analyzed for each experimental condition and the responses were count data not normally distributed. Alternatively, two not parametric statistic tests described by Moodie and colleagues [Moodie et al., 2006], called distribution free resamplig (DFR(eq) and DFR(2x)) could also be used for our study, but our choise fell on the empirical evaluation.

One of the empirical rules described by Moodie and co-workers [Moodie et al., 2010] was used for the interpretation of the ELISpot results: a response was considered positive when a difference of more than twofold between the spot counts in the experiment versus those in the background wells was observed. Applying this rule, no minimum spot number is required. The second empirical rule decribed by the authors defines a positive response on the basis of a threshold of 5 spots per 100,000 PBMCs, in the experiment wells and an increase of two fold in the spot number over background. This second rule could also be applied in our evaluation, since we always observed more than 5 spots in the experiment wells and, in addition, the quantity of effectors plated was less than that reported (10,000/well). The advantage of this second rule is the lower percentage of false positive rate compared to the first empirical rule (3% and 17% respectively). A second important factor to consider is the limit of detection

of the ELISpot assay. Generally, the limit of detection is defined as the lowest amount of analyte which can be detected in a sample but not necessary quantified as an exact value. For the ELISpot analysis a signal-to-noise approach, that compares the spot count in the experiment wells (signal) with the spot counts in the medium control wells (noise) could be applied. The ratio between signal and noise must be comprised between 2:1 to 3:1. However, to increase the power of both empirical and statistical tests, it would be ideal to have more replicate wells for both control and experimental condition. It has been demonstrated that even an increased number of the replicates for the controls would already increase the power to detect an immune response [Hudgens et al., 2004, Moodie et al., 2006]. Anyway, at present, the number of effectors obtained after co/micro-culture not allows to add further control wells.

Taken together these results showed that, although our immunological study must be done on an increased number of CRC patients, and the CTLs lytic ability against autologous tumor cells must be performed, it can be hypothesized that the generation of tumor-specific CTLs activated *ex-vivo* against tumor could be useful for supporting ACT in CRC.

6. CONCLUSION

ACT has been developed as a therapeutic approach for tumors arising from the hematopoietic system, such as acute and chronic myeloid leukemia, lymphoma and multiple myeloma [Montagna et al., 2001].

Subsequent literature studies published the use of a similar strategy also for solid tumors, such as melanoma and RCC, two types of neoplasia that appear exceptional in their ability to stimulate spontaneously the anticancer endogenous immune cells [Kurokava et al., 2001].

The process of tumor progression involves complex interactions among different cells of the immune system and different effector molecules. The effector T lymphocytes are the key players that prevent the tumor development and inhibit the tumor progression [Dunn et al, 2004]. The PBMCs isolated from the blood of RCC patients were stimulated *in vitro* with tumor-loaded DCs. A primary T-cell response against RCC was elicited and expansion of tumor-specific CTLs for adoptive transfer was achieved. It was demonstrated that restimulation with autologous irradiated tumor cells alone was optimal for the induction of tumor specific CTLs *in vitro*. These $CD8^+$ T-cells exhibited strong MHC class I-restricted cytotoxic activity against the autologous tumor [Kurokava et al, 2001].

In our study a new immunotherapeutic approach for CRC was developed.

The interest of the scientific community for this tumor is very high, because it is one of the most common diagnosed cancers wordwide. In addition, although CRC mortality has decreased during the last years, it remains the third cause of cancer related mortality. Improving methods of diagnosis and therapeutic possibility for CRC patients are objectives to be achieved.

The adoptive immunotherapeutic approach proposed in our study was focused on: 1) the establishment of primary colon cell lines from colon biopsies; 2) the obtainment of DCs from monocytes and of T lymphocytes

by means of PBMCs magnetic labeling; 3) the activation of autologous anti-tumor CTLs in co/micro-culture experiments.

The evaluation of the T-cells activation was performed using an ELISpot assay specific for the IFN-γ production. The biological functions of this cytokine include both direct cytotoxic antiproliferative effects on tumor cells and stimulation of adaptive immune system cells against tumor antigens.

Our protocol suffered of some limitations that should be avoided: first of all, often the biological speciemens were not enough to perform the immunological confirmatory evaluations to set up further controls. For example, the IFN-γ secretion after co/micro-culture stimulation would have been evaluated by ICS. In fact, by evaluating also the cell surface markers, IFN-γ secretion could be confered to CTLs with absolute certainty. Similarly, even if the absence of NK cells in co/microculture was confirmed by flow cytometry, additional ELISpot controls should established with effector cells cultured with the leukemic cell line K562, in order to exclude the IFN-γ production by contaminant NK cells. Additionally, another ELISpot control should be composed by the effector cells cultured in presence of DCs not loaded with tumor antigens.

Although our results suggested that CTLs activated *ex vivo* against the tumor could be useful for supporting the ACT protocols, two main aims need be achieved in the near future, to complete this study and to validate its scientific relevance as new therapeutic strategy of CRC.

The first aim is the CTLs expansion: the effector cells that secreted IFN-γ were criopreserved for this purpose. We are planning to perform the CTLs expansion following previous published protocols. Briefly, after tumor-specific stimulations, CTLs will be further expanded in presence of autologous irradiated feeder cells, OKT3 and of a low dose of rh IL-2. The rapidly expanded cultures will be maintained for about 15 days. After the

first round of antigen-independent expansion, specificity and sterility tests will be performed to confirm the quality of the product, and then a second cycle of expansion will be conducted. At the end of the process and before cryopreservation, CTL lines will be tested again for their cytotoxic activity against autologous tumor and for microbial contaminations.

The second aim is the assessment of the cytotoxic activity of the CTL lines against autologous tumor (CTLs should be able to lyse patient tumor cells). We are planning to use for this analysis a 51Chromium release assay, or alternatively a non-radioactive assay based on the measurement of lactate dehydrogenase, which is released upon cell lysis in the same way as 51Chromium is released. Target cells will include autologous tumor cells, K562, while non-malignant control cells will include autologous T and B-lymphoblastoid cell lines and autologous fibroblasts, for evaluating the auto-aggressive potential of CTLs. Preincubation of target cells with MoAb anti-HLA Class I and Class II will be used to assess the specificity of tumor cells killing.

After the achievement of these objectives, the experimental design could be transferred to a GMP laboratory to ensure the safety of the autologous CTLs activated *ex-vivo* against tumor and their re-infusion in CRC patients.

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Scientific productions relative to this work

Conference attendance as selected speaker:

“10° Meeting Nazionale del NIBIT” November, 5-7 2012: Castelnuovo Berardenga, Siena, Italy “Generation of tumor specific cytotoxic T-lymphocytes suitable for adoptive immunotherapy from peripheral blood of advanced colorectal patients”.

Abstracts accepted at national and international conferences:

- “XIV Congresso Nazionale di Oncologia Medica” Roma, 27 - 29 ottobre 2012 “Generation of tumor-specific cytotoxic T-lymphocytes suitable for adoptive immunotherapy from peripheral blood of advanced colorectal cancer patients” **Silvia Carluccio**, Serena Delbue, Pasquale Ferrante, and Marco Bregni.

- "2013 Gastrointestinal Symposium" - San Francisco, California, January 24-26, 2013 "Generation of tumor-specific cytotoxic T-lymphocytes suitable for adoptive immunotherapy from peripheral blood of colorectal cancer patients" **Silvia Carluccio**, Serena Delbue, Pasquale Ferrante, and Marco Bregni.

- “EBMT 2013” - London UK, April 7-10, 2013 "Generation of tumor-specific cytotoxic t-lymphocytes from peripheral blood of colorectal cancer patients for adoptive T-cell transfer" **Silvia Carluccio**, Serena Delbue, Pasquale Ferrante, Marco Bregni

- "2013 ASCO Annual Meeting" - Chicago, May 31 - June 4, 2013 "Generation of tumor-specific cytotoxic T-lymphocytes suitable for adoptive immunotherapy from peripheral blood of colorectal cancer patients" **Silvia Carluccio**, Serena Delbue, Pasquale Ferrante, Andrea Galli, Alberto Della Valle, Marco Bregni.

- “4th International Satellite Symposium AICC–GISM” Brescia 20-22 November 2013 “Adoptive immunotherapy in colon cancer: generation of tumor-specific cytotoxic T lymphocytes (CTLs) from peripheral blood of colorectal cancer patients” **Silvia Carluccio**, Serena Delbue, Pasquale Ferrante, Lucia Signorini, Simone Dallari, Francesca Elia, Andrea Galli, Alberto Della Valle, Marco Bregni

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ACKNOWLEDGMENT

This project was founded by the Umberto Veronesi Foundation (GRANTS 2012).

I would like to thank Dott. Marco Bregni for his scientific support and all the clinicians for patients enrollement.