UNIVERSITÀ DEGLI STUDI DI PADOVA

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The role of immune surveillance mechanisms acting to prevent an AOM - induced colorectal carcinogenesis progression

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Abbreviations

Abbr.	Term
5-FU	5-fluorouracil
ACF	Aberrant crypt foci
AOM	Azoxymethane
AP-1	Activator protein 1
APCs	Antigen-presenting cells
ATM	Ataxia-telangiectasia mutated kinase
ATR	ataxia telangiectasia and Rad3-related protein
CD4+	T helper cells
CD8+	Cytotoxic T cells
CEC	Colonic epithelial cells
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability pathway
CMSs	Consensus molecular subtypes
CpG	Cytosine preceding Guanine
CRC	Colorectal cancer
CTL's	Cytotoxic T cells
Ctla4	Cytotoxic T-lymphocyte protein 4
CXCL/CCL	Chemokine motif ligands
DAMPs	Damage-associated molecular patterns
DCC	Deleted in colorectal cancer
DCs	Dendritic cells
DDR	DNA damage response
DMH	Dimethylhydrazine
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ENE	Extra-nodal extension
ENU	Eyhylnitrosurea
FAP	Familial adenomatous polyposis
FDA	Food and drug administration
GPX	Gluthathione peroxidases
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferases
HGD	High grade dysplasia
HIF1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
HNF4A	Hepatocyte nuclear factor 4α

HNPCC	Hereditary non-polyposis colorectal cancer
iDCs	Immature dendritic cells
IDO	Indolamine-2,3-dioxygenase
IECs	Intestinal epithelial cells
IFNs	Interferons
IGF2	Insulin-like growth factor 2
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Irinotecan
IRF	Interferon regulatory factor
IRS2	Insulin receptor substrate 2
JNK	c-Jun-N-terminal kinase
LGD	Low grade dysplasia
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
LV	Leucovorin
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MMR	Mismatch repair
MSI	Microsatellite instability
NAC	N-acetyl cysteine
NADPH	Nocotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFE2	Nuclear factor erythroid-2
NK	Natural killer cells
NKG2D	Natural Killer Group 2D receptor
NO	Nitric oxid
Nrf2	Nuclear factor related factor-2
OX	Oxaliplatin
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed death-1
PDL-1	Programmed death-1 ligand
PDL-2	Programmed death-2 ligand
PI3K	Phosphoinositide 3-kinase
PPARγ	Peroxisome proliferator-activated receptor gamma
PPRs	Pattern recognition receptors
PRXs	Peroxiredoxins
RNS	Reactive nitrogen species
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species

SCFA	Short Chain Fatty Acids
SODs	Superoxide dismuteses
Sp-1	Specifity protein-1
STAT3	Signal transducer and activator of transcription 3
T reg	Regulatory T cell
TAAs	Tumor associated antigens
TCR	T-cell receptor
TDM	Therapeutic drug monitoring
TGFβ	Transforming growth factor beta
Th	T helper
TIM-3	T cell immunoglobulin domain and mucin domain-3.
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TSGs	Tumour suppressor genes
TSGs	Tumor suppressor genes
UC	Ulcerative colitis

1 Abstract

Colorectal cancer (CRC) is one of the most frequent cancer worldwide both in men and women. This tumour is the result of a multistep process that includes mutations of proto oncogenes and tumour suppressor genes, in addition to epigenetic modifications. In this well-characterized process of cancer progression, the immune system mechanisms have a central role.

The closed connection between the tumour development and immune system is the so-called *immune surveillance*, a dynamic process that comprises three different steps: elimination, equilibrium and escape. In the elimination phase our immune cells attempt to eliminate single transformed cells in order to avoid cancer development. When the immune system fails to identify and to eliminate these cells, the organism enters in the following phase called equilibrium in which cancer cells persist in the tissue in a low but relatively stable number. If the organism fails to maintain this phase of balance, tumoral cells can expand in the organism.

Accordingly, it is known that the progression from pre-neoplastic lesions to invasive cancer is not common, suggesting that the underlying immune surveillance mechanisms must be highly effective. In particular, my research group has previously described how in patients with ulcerative colitis (in which the cumulative risk of colon cancer is lower than the actual rate of dysplasia) the co-stimulatory molecule CD80 is overexpressed in dysplastic colonic mucosa. Moreover, we reported that the effective co-stimulation given by CD80 signalling between intestinal epithelial cells and T-cells controls the progression from low to high grade dysplasia in a mice model of inflammatory colonic carcinogenesis.

Besides, the microbiota signalling has a pivotal role in intestinal colon cancer carcinogenesis. In particular, Toll-like receptors that are a family of pattern recognition receptors that have a key role in the first line defence against pathogens, seem to regulate a wide range of biological responses including inflammatory and immune responses during carcinogenesis. In detail, TLR4 signalling has been involved in the regulation of tumour growth, survival and progression connected to inflammation but its role is still controversial in non-inflammatory carcinogenesis.

Thus, the working hypothesis of my PhD project was that CD80 expression on dysplastic epithelial cells is crucial also in successful immune surveillance of sporadic colorectal cancer (CRC). Therefore, the aim of my work was to examine the role of CD80 expression in epithelial cells during colonic carcinogenesis combining analysis of human tissues, *in vivo* animal studies and *in vitro* experiments.

The analysis of human colonic surgical specimens demonstrated that CD80 is over-expressed by epithelial cells in colonic pre-neoplastic lesions. Accordingly, also in our azoxymethane (AOM) induced colonic adenocarcinoma model, CD80 is overexpressed by dysplastic epithelial cells supporting a key role of this molecule in the early phases of the carcinogenesis process. Furthermore, the lack of functional CD80 in colonic mucosa accelerated the progression of colonic carcinogenesis. Remarkably, the use of $CD80^{ko}$/WT bone marrow chimeras in the AOM CRC model further demonstrated the contribute of epithelial CD80 expression to inhibition of dysplasia development. Overall, our results suggest that CD80 expression is induced in preneoplastic lesions as a protective mechanism against AOM-induced epithelial transformation.

Since AOM-induced carcinogenesis is associated to oxidative stress we hypothesized that CD80 expression is upregulated in intestinal epithelial cells by reactive oxygen species (ROS). Accordingly, our *in vitro* experiments with intestinal epithelial CT26 and primary intestinal epithelial cells suggested that oxidative stress has a prominent role in CD80 induction. Moreover, the thoughtful investigation of CD80 up regulation induced by ROS revealed that ROS induce CD80 expression via MAPK pathways that activate STAT3 transcription factor in colon epithelial cells.

In conclusion, in this first part of my study we showed that CD80 is crucial in the early stages of sporadic colorectal carcinogenesis and free radicals could have a prominent role in both the mechanisms of carcinogenesis and immune surveillance triggering the immune response.

In the second part of my PhD work we investigated the role of TLR4 signalling in the AOM induced CRC model.

Along with the histological results showing that invasive carcinoma was more frequent in TLR4 deficient mice ($TLR4^{KO}$) compared to Wild Type (WT) mice both

sacrificed after 8 months from the first AOM injection, flow cytometric analysis revealed that MHC expression is impaired in intestinal epithelial cells of AOM-treated TLR4 deficient mice. Accordingly, the percentage of CD8+ cytotoxic and CD4+ helper T lymphocytes decreased in AOM-treated TLR4 deficient mice proposing a less efficient presentation of the tumoral antigens and the presence of a more effective immune escape mechanism compared to the WT mice.

Since microbiota plays a key role to modulate immune cells activities we next compared the gut microbiota of WT and TLR4 deficient mice by 16S rDNA sequencing and analysis of faecal SCFA (Short Chain Fatty Acids) by GC-MS. Our results showed that WT and TLR4 deficient mice display a comparable composition of the microbiota, thus excluding that the differences observed in AOM-induced CRC development are secondary to microbiota differences, generating anomalous signals to mucosal immune cells.

Indeed, *in vitro* experiments with bone marrow derived DCs confirmed the absolute requirement of TLR4 signalling to generate mature and competent DCs.

Concluding, we showed that TLR4 signalling is protective in sporadic colorectal carcinogenesis enhancing the immune response against tumour cells. This result improves the understanding of TLR4-targeted applications, its role in tumor progression and its potential use as immune modulating agent.

1 Riassunto

Il cancro al colon-retto (CRC) è uno dei tumori più frequenti in tutto il mondo sia negli uomini che nelle donne. Questo tumore è il risultato di un processo multistep che include mutazioni di protoncogeni e geni oncosoppressori, oltre che a modificazioni epigenetiche. In questo processo ben caratterizzato di progressione del cancro i meccanismi del sistema immunitario svolgono un ruolo centrale.

Lo stretto legame tra lo sviluppo del tumore e il sistema immunitario è la cosiddetta sorveglianza immunitaria, un processo dinamico che comprende tre diversi passaggi: eliminazione, equilibrio e fuga. Nella fase di eliminazione il nostro sistema immunitario tenta di identificare ed eliminare le cellule trasformate per evitare lo sviluppo di focolai di cellule neoplastiche. Quando il sistema immunitario fallisce nell'eliminare queste cellule, l'organismo entra nella fase successiva chiamata equilibrio in cui le cellule tumorali persistono nel tessuto in un numero basso ma relativamente stabile. Se l'organismo non riesce a mantenere questa fase di equilibrio, le cellule tumorali possono espandere la massa tumorale e diffondersi nell'organismo (fase di fuga).

È risaputo che la progressione dalle lesioni pre-neoplastiche al cancro invasivo non è comune, cosa che suggerisce come i meccanismi di sorveglianza immunitaria sottostanti siano altamente efficaci. In particolare, il mio gruppo di ricerca ha precedentemente descritto come nei pazienti con colite ulcerosa (in cui il rischio cumulativo di carcinoma del colon è inferiore al tasso effettivo di displasia) la molecola co-stimolante CD80 sia sovraespressa nella mucosa displastica del colon. Inoltre, in un modello murino di carcinogenesi infiammatoria del colon abbiamo dimostrato che la costimolazione tra le cellule epiteliali intestinali e le cellule T mediata dal CD80 controlla la progressione da basso grado ad alto grado di displasia.

Inoltre, il microbiota ha un ruolo chiave nella carcinogenesi del cancro al colon, ruolo svolto probabilmente attraverso i recettori Toll-like, una famiglia di recettori con un ruolo chiave nella prima linea di difesa contro i patogeni, che sembrano regolare un'ampia gamma di risposte biologiche incluse le risposte infiammatorie e immunitarie durante la carcinogenesi. Nel dettaglio, il TLR4 è coinvolto nella regolazione della

crescita, della sopravvivenza e della progressione tumorale connesse all'infiammazione, ma il suo ruolo è ancora controverso nella carcinogenesi non infiammatoria.

Pertanto, il primo obbiettivo del mio progetto di dottorato è stato di dimostrare che l'espressione di CD80 sulle cellule epiteliali displasiche è cruciale nel successo della sorveglianza immunitaria del cancro colorettale sporadico. A tal scopo abbiamo esaminato l'espressione del CD80 nelle cellule epiteliali del colon di tessuti ottenuti da resezioni chirurgiche, utilizzato un modello murino basato sull'uso dell'azossimetano (AOM) per indurre la carcinogenesi ed eseguito esperimenti *in vitro.*

I dati ottenuti dall'analisi dei pazienti dimostrano che il CD80 è significativamente sovraespresso dalle cellule epiteliali nelle lesioni pre-neoplastiche ma si riduce quando si è sviluppata una neoplasia invasiva. In maniera analoga, il CD80 è sovraespresso nelle prime fasi del processo di carcinogenesi nei topi trattati con AOM. Inoltre, la mancanza del CD80 funzionale nella mucosa accelera la progressione della carcinogenesi nel nostro modello murino e l'uso delle chimere supporta come l'espressione epiteliale di CD80 contribuisca all'inibizione dello sviluppo della displasia. Complessivamente questi dati confermano il ruolo chiave del CD80 all'inizio del processo tumorale.

Nel loro insieme, i nostri risultati suggeriscono che l'espressione di CD80 è indotta nelle lesioni preneoplastiche come meccanismo protettivo contro la degenerazione epiteliale indotta da AOM. Utilizzando la linea cellulare epiteliale intestinale CT26 e le cellule epiteliali intestinali primarie da topo, abbiamo accertato che lo stress ossidativo ha un ruolo fondamentale nell'induzione dell'espressione di CD80. Inoltre, i ROS nelle cellule epiteliali del colon inducono l'espressione di CD80 attraverso le vie attivate dalle MAP chinasi, le quali mediano la fosforilazione di STAT3.

In conclusione, nella prima parte del mio progetto abbiamo dimostrato che il CD80 è fondamentale nelle fasi iniziali della carcinogenesi sporadica del cancro al colon e che i ROS hanno un ruolo cruciale sia nei processi di carcinogenesi che di attivazione della sorveglianza immunitaria.

Nella seconda parte del mio progetto di dottorato, abbiamo messo in luce il ruolo protettivo dei segnali derivati dal recettore TLR4 nel modello di cancro al colon indotto da azossimetano. Infatti, l'analisi istologica ha dimostrato una maggiore incidenza di

carcinoma colico invasivo nei topi TLR4^{KO} rispetto ai topi Wild Type sacrificati dopo 8 mesi la prima iniezione AOM. Inoltre, l'analisi citofluorimetrica ha rivelato che l'espressione del complesso maggiore di istocompatibilità I e II era alterata nei topi TLR4^{KO}, e parallelamente la percentuale di linfociti T helper CD4+ e citotossici CD8+ era diminuita significativamente. Questi dati suggeriscono una minore presentazione degli antigeni tumorali da parte delle cellule epiteliali favorendo i meccanismi di fuga da parte delle cellule tumorali nei topi TLR4^{KO}.

Abbiamo quindi determinato il livello di espressione di specifici marcatori molecolari dello sviluppo e della maturazione delle cellule dendritiche nella mucosa colica. Questi saggi hanno dimostrato nei topi TLR4^{KO} un ridotto livello di cellule dendritiche mature critiche per l'attivazione delle cellule T antitumorali.

Poiché il microbiota svolge un ruolo chiave nel modulare le attività delle cellule immunitarie, abbiamo confrontato il microbiota intestinale dei topi WT e TLR4^{KO} mediante il sequenziamento dell'rDNA 16S e abbiamo quantificato gli acidi grassi a corta catena (SCFAs), prodotti metabolici del microbiota intestinale, attraverso la GC-MS. I nostri risultati hanno mostrato che i due gruppi hanno una composizione comparabile del microbiota, confermando quindi che le differenze osservate nello sviluppo del CRC indotto da AOM non siano secondarie alla composizione del microbiota.

Esperimenti in vitro utilizzando cellule dendritiche isolate dal midollo osseo hanno confermato la necessità del segnale proveniente dal TLR4 per ottenere cellule dendritiche mature e competenti.

Concludendo, abbiamo dimostrato che il recettore TLR4 svolge un ruolo protettivo nella progressione della carcinogenesi colorettale sporadica, migliorando la risposta immunitaria contro le cellule tumorali. Questi dati aiutano a migliorare la comprensione del ruolo del TLR4 nelle neoplasie coliche ed il suo potenziale utilizzo come agente immunomodulante.

2 Introduction and background

Colorectal Cancer (CRC) is the third most frequent type of cancer worldwide [1]. The GLOBOCAN series of the International Agency for Research on Cancer estimates the worldwide incidence and mortality of 27 major cancers and for all cancers combined for 2012. Lung (1.82 million), breast (1.67 million), and colorectal (1.36 million) are the most commonly diagnosed cancers [2,3].

In terms of incidence, CRC counts 1.4 million cases and 694,000 deaths. It is the third commonest cancer (cancer incidence) in men with around 750,000 cases (10% of the total) and the second commonest in women with about 610,000 cases (9,2% of the total) [3].

More than half of the cases occur in more industrialised countries. There is wide-ranging geographical variation in incidence through the world and mortality is lower (694,000 deaths, 8.5% of the total) with more deaths (52%) in the less developed regions of the world, indicating a poorer survival in these areas. The highest estimated mortality rates for both sexes is in Central and Eastern Europe (20.3 per 100,000 for men, 11.7 per 100,000 for women), whereas the lowest is in Western Africa (3.5 and 3.0, respectively) [3].

For several decades, the basis for systemic treatment of advanced colorectal cancer has been chemotherapy: 5-fluorouracil (5-FU) in combination with leucovorin (LV). More recent agents, such as irinotecan (IR) and oxaliplatin (OX), have improved the response and survival rates. The administration of pre-operative chemotherapy to patients with initially non-resectable CRC can reduce the size of the tumours such that curative resection becomes possible and can also reduce the risk of recurrence following resection. Although several mechanisms determine the action of conventional chemotherapy, they all seek to act on the tumour cells, blocking the replication of their DNA. As they are not specific for the cancer cells, these mechanisms are often associated with toxicity for normal tissues [4].

Endoscopic or surgical resection is common used for early premalignant adenomas and for the treatment of the most early stages carcinomas [5].

Liver metastases of CRC are very common (about 50% of patients) and this is a frequent cause of CRC-related deaths [5]. Nevertheless, the survival of CRC affected people is connected to the stage at the moment of the diagnosis but the 5-year survival rate for patients with metastatic CRC is less than 10% [6].

2.1 Colorectal cancer pathogenesis

CRC presents in three major forms: inherited, sporadic and familial. Although the mechanisms underlying familial CRC are poorly comprehended, a large body of experimental evidences suggests that inherited and sporadic CRC are caused by sequential genetic and molecular events [7].

Ten percent of CRC are inherited and presents as well-characterized cancer predisposition syndromes including Lynch syndrome and familial adenomatous polyposis (FAP). Familial CRC accounts for 25% of CRCs and presents without precisely defined Mendelian inheritance patterns or genetic aetiology. Finally, sporadic CRC (approximately 70% of CRCs cases) derives from somatic mutation(s) and is not associated with family history [7].

Most CRCs progress through a series of morphological stages (Fig. 1). In the first histological signs, one or more crypts show accumulation of excess cells at the surface. The cells in aberrant crypt foci may appear normal, forming hyperplastic tissue, or they may have irregular intracellular and intercellular organization, forming dysplastic tissue. As excess cells concentrate, visible polyps develop and protrude from the epithelial surface. If the polyp is dysplastic, the tumour is called adenoma. Adenomas tend to become more dysplastic as they grow [8].

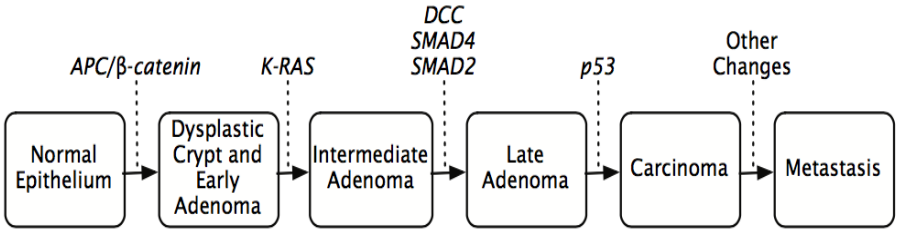


Fig. 1. *Morphology of colorectal cancer progression [8].*
Approximately 50–85 % of colorectal cancers follow this pathway.

Colorectal cancers originate through a multistep process that consist of genetic and epigenetic alterations (Fig. 2). In particular, three different pathogenetic pathway have been involved:

- Chromosomal instability pathway (CIN): a sequential deregulation of tumour suppressor genes (TSGs) and oncogenes such as, *APC*, *KRAS*, *DCC/SMAD4*, and *TP53*. It generally occurs within inherited tumours, such as familial adenomatous polyposis (FAP), but it has also been associated with the majority of sporadic CRCs [9].

  The initial mutation in most of the cases appears at the APC tumour-suppressor gene locus (5 q21 – q22) with the loss of its function. Usually the APC regulatory pathway represses β-catenin that results in reduction of tendency for abnormal tissue expansion and preventing excessive cells proliferation. Additionally, APC is involved in the apoptosis regulation, cell – cycle progression and chromosomal stability [10–14]. Disruption of the APC pathway may be sufficient to start a small adenomatous growth by allowing unregulated activation of Wnt signalling involved in the down regulation of β-catenin [15–18]. Moreover, hyperactivation of the Wnt signalling is critical to the initiation and the maintenance of the majority of CRCs [19,20]. The Wnt pathway is also fundamental to preserve the intestinal epithelium that is renewal every 4-5 days so it is not possible to target this signalling in cancers without disturbing intestinal regeneration [21,22]. About hundreds of specific APC mutations have been described, and the particular position of the mutation can dictate the severity and the onset of the hereditary syndrome FAP. In fact, patients with FAP have an autosomal dominant inherited germline mutation of APC, consequently they are more predisposed to mutation of the remaining wild type APC allele. FAP is characterized by the presence of a huge number of polyps in the large bowel that extend first in the rectum and distal colon before arising to proximal segments. Most of FAP patients will develop CRC in distal colon [23,24].

  Mutation of a RAS gene often occurs among the next genetic events of progression and it acts oncogenically, with a mutation to a single allele sufficient to cause progression. As adenomas continue to grow and begin to show great histological

abnormalities, they tend to lose parts of 18q (the long arm of chromosome 18). Also, the genes DCC, SMAD4 and SMAD2 in 18q21 play a role in carcinogenesis [25].

Thus, loss of the gene "deleted in colorectal carcinogenesis " DCC (a surface protein with extensive homology to other cell adhesion) often occurs in cancer, in fact it is deleted in more than 70% of CRC cases [26,27]. SMDA4 and SDMA2 may interact with the transforming growth factor beta (TGFβ) pathway, that often suppresses normal cellular growth. The TGFβ family are known inhibitors of gastrointestinal epithelial cells proliferation and usually they are involved in the phosphorylation of the previously cited proteins SMAD2 and SMAD3 that form a heteromeric complex with SMAD4 which translocates in the nucleus inducing TGFβ target gene transcription [17,28,29].

Transition to cancer is connected with loss of functional p53 [17,30] by damage to both alleles, abolishing suppression of cell division or apoptosis in response to stress or damage [8]. In fact, p53 protein is called "guardian of the genome" because it is involved in several basal cell functions, such as cell cycle, DNA repair and apoptosis. It also acts as transcription factor promoting the expression of genes connected to growth inhibition. The half-life of wild type p53 is around 20 minutes whereas for the p53 mutant is approximately 24 hours. For this reason mutant p53 could accumulate in the nucleus and is over expressed in tumours [31–33]. The presence of mutant p53 is a common event in tumours [33].

Genomic studies have also shown that alterations in the WNT–β-catenin, transforming growth factor-β (TGFβ), EGFR and downstream MAPK and PI3K signaling pathways are nearly ubiquitous events in CRC [25].

Overall, the chromosomal instability (CIN) is observed in 70%–85% of CRCs [34]. The most frequently mutated genes in CRC are listed in Table 1.

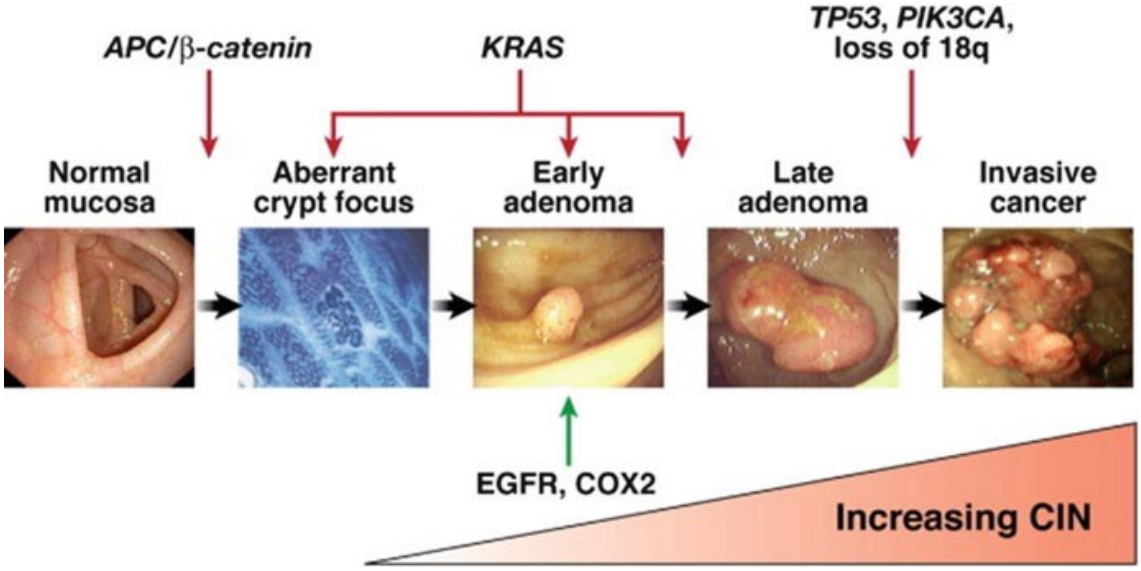


Fig. 2. *The adenoma–carcinoma sequence* [35].
The initial step in colorectal carcinogenesis is thought to be the formation of aberrant crypt foci (ACF). Activation of the Wnt pathway occurs during this step as a result of inactivating mutations in the APC gene. Progression to adenoma and carcinoma is usually mediated by activating mutations in KRAS and loss of TP53 expression, respectively. A subset of advanced adenomas may progress due to mutations in PIK3CA and loss of 18q [35].

Table 1. *Some of the most frequently mutated genes in CRC.*
Adapted from R. E. McIntyre et al. DOI 10.1002/bies.201500032 [36] .

Human/mouse gene symbol	Frequency (%)	Full gene name	Role in CRC development
APC/Apc	>80	Adenomatous Polyposis Coli	- Inactivation of APC is the initiating event in the majority of CRC - APC in a negative regulator of the Wnt pathway - hyperactivation of the Wnt pathway initiates development of CRC by stabilising the β-catenin transcription factor
TP53/Trp53	~65	Tumour protein p53	- loss of Tp53 is associated with disease progression - TP53 regulates expression of genes that induce cell cycle arrest, apoptosis, senescence, DNA repair
KRAS/Kras **BRAF/Braf**	~45 ~8	Kirsten rat sarcoma viral oncogene homolog v-raf murine sarcoma viral oncogene homolog	- Mutation of KRAS and BRAF cause activation of the Ras – MAPK pathway - Activation of the Ras – Mapk pathway is associated with disease progression and poor prognosis - Activation of the Ras – Mapk pathway leads to activation of transcription factors such as c-Myc and c-Fos which in turn regulate expression of cell cycle genes
TGFBR2/Tgfbr2 **SMAD2/Smad2**	~12 ~7	Transforming growth	- Alterations in TGFb pathway e.g. mutation of TGFBR2, SMAD2, SMAD3 and SMAD4, are

SMAD3/Smad3 **SMAD4/Smad4**	~5 ~12	factor, beta receptor 2 SMAD family member 2, 3, 4	associated with disease progression - The SMAD family are intracellular signal transducers that are activated by TGFb receptors and act as transcriptional modulators.

- An alternative pathways that leads to CRC is the microsatellite instability (MSI), responsible for the Lynch syndrome and sporadic tumours and is mainly caused by inactivation of the DNA mismatch repair genes (*hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2*) [9] (Fig. 3). Loss of MMR (mismatch repair) causes increased mutation in repeated DNA sequences, such as those in microsatellites regions. This failure to repair mismatches causes repetitive microsatellites to change their length at a much higher rate than normal during DNA replication [8,37–39].

  MSI is responsible for approximately 15-20 % of all CRC cases [34]. These tumours contain a germline mutation in one of the MMR genes, followed by a second hit to the wild-type copy (inherited from the unaffected parent); this could occur via loss of heterozygosity (LOH), methylation, or point mutation. Defects in MMR result in MSI and rapid accumulation of somatic mutations. It has been proposed that tumours arise via mutations in a few critical genes, but that large numbers of microsatellite mutations also occur, most of which are simply passengers that provide the mutational signature used to identify tumours with MSI (Fig. 3). Colorectal tumours that develop in patients with Lynch syndrome frequently have mutations in KRAS. Most cases of CRC associated with MSI are not inherited (familial) but occur through sporadic methylation-induced silencing of MLH1. These sporadic tumours have the CpG island methylator phenotype (CIMP) signature, resulting in methylation of many gene promoters. When the MLH1 promoter is methylated, MMR activity fails and MSI ensues. Thus, the mutational signature of sporadic tumours includes CIMP and MSI. BRAF mutations are also observed in most sporadic colorectal tumours, but do not occur in tumours of patients with Lynch syndrome [40].

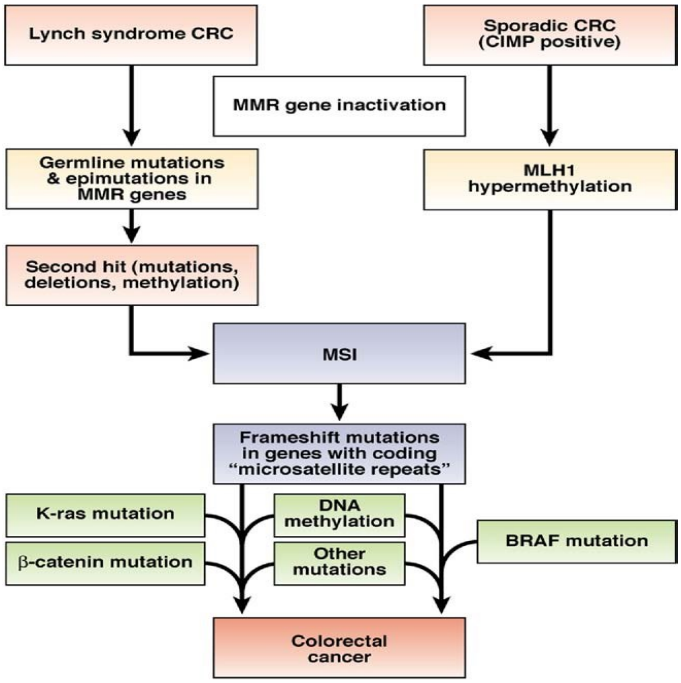


Fig. 3. *The two molecular pathways to the development of CRC with MSI* [40].

- CpG island methylator phenotype (CIMP), hypermethylation of CpG islands in the promoter region of a gene often impairs the ability of transactivating factors to bind and initiate a gene transcription [41]. CpG (cytosine preceding guanine) islands are regions within the genome that are common in promoter sites rich in CpG dinucleotides. More than 50% of human genes have been found to be regulated in this way, by promoters including CpG islands. Several CpG dinucleotides, which are methylated in normal cells, are unmethylated in cancer. In cancer cells, CpG islands may also be aberrantly hypermethylated, causing inappropriate silencing of gene expression. *p14, p16, hMLH1, MGMPT,* and *HPP1 are c*ommonly hypermethylated genes in colorectal cancers [8].

2.2 The consensus molecular subtypes of colorectal cancer

To facilitate the clinical translation of the gene expression-based CRC classifications above described it's commonly used the so-called Consensus Molecular Subtypes (CMSs) with different features: CMS1 (microsatellite instability immune, 14%) microsatellite unstable, hypermutated and strong immune activation; CMS2 (canonical, 37%), epithelial, marked Wnt and MYC signaling activation, chromosomally unstable; CMS3 (metabolic, 13%), epithelial and evident metabolic dysregulation; and CMS4

(mesenchymal, 23%), conspicuous transforming growth factor activation, stromal invasion and angiogenesis [42].

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Fig. 4. *Taxonomy of colorectal cancer, with significant biological differences in the gene expression-based molecular subtypes [42].*
CpG island methylator phenotype (CIMP); microsatellite instability (MSI); somatic copy number alterations (SCNA).

Thus, this comprehensive correlative analyses with well-defined genomic and epigenomic CRC characteristics enabled deeper understanding of the biological features of each CMS. Tumors with MSI cluster in the CMS1 group (MSI immune subtype, 14% of early-stage tumors) are characterized by hypermutation, hypermethylation and strong infiltration of the tumor microenvironment with immune cells, particularly CD8+ cytotoxic T lymphocytes, CD4+ T helper 1 cells and natural killer cells [25].

Tumors with CIN can be subclassified into three groups on the basis of gene expression signals:

1. CMS2 (canonical subtype, 37% of early-stage tumors);
2. CMS3 (metabolic subtype, 13% of early-stage tumors);
3. CMS4 (mesenchymal sub- type, 23% of early-stage tumors).

CMS2 and CMS4 cannot be distinguished by their somatic copy number alteration patterns and mutations, in fact both groups present microsatellite stability (MSS) and low levels of gene hypermethylation. However, CMS2 epithelial tumors have marked upregulation of Wnt and MYC downstream targets, higher expression of the oncogenes

EGFR, *ERBB2* (also known as *HER2*), insulin-like growth factor 2 (*IGF2*), insulin receptor substrate 2 (*IRS2*) and transcription factor hepatocyte nuclear factor 4α (*HNF4A*), as well as cyclins. CMS4 tumors are characterized by pathways related to epithelial–mesenchymal transition (EMT), TGFβ and integrins, and show marked overexpression of proteins implicated in extracellular matrix remodeling and complement signaling. CMS4 tumors exert a proangiogenic and stroma genic influence on the microenvironment. Thus, the considerable differences in pathway activation between CMS2 and CMS4 tumors traduce into significantly higher risk of relapse and death for patients diagnosed with early-stage CMS4 mesenchymal CRC [25,42].

To conclude, CMS3 tumors have a distinctive genomic and epigenomic characterization as compared with other CIN groups, with consistently fewer copy number alterations. The dominant feature at the pathway level of CMS3 epithelial tumors is metabolic reprogramming, including activation of glutaminolysis and lipogenesis. In addition, CMS3 tumors are enriched for *KRAS*-activating mutations, which have been linked to prominent metabolic adaptation in CRC and other malignancies [25,42].

2.2.1 Inflammatory bowel diseases (IBDs) and CRC

CRC risk is increased in inflammatory bowel disease (Crohn's disease and ulcerative colitis), but the mechanisms are not well established. Inflammation may increase mutagenesis via generation of oxidative stress and free radicals that may promote proliferation of colorectal epithelial cells [7]. Ulcerative colitis (UC) is a chronic inflammatory disorder involving the rectum and to a various extent the colon. UC patients experience several complications including an increased risk of colorectal cancer (CRC) [43–46].

Colitis associated colon cancer develops in a stepwise well characterized progression from inflamed tissue through low grade and high-grade dysplasia to invasive carcinoma. In fact, in UC, oxidative DNA damage and DNA methylation are the early events that can produce inhibition of oncosuppressor genes, mutation of p53, aneuploidy and microsatellite instability. Hypermethylation of tumour suppressor and DNA MMR gene promoter regions is an epigenetic mechanism of gene silencing that can be involved in tumorigenesis and may also represent the first step in inflammatory

carcinogenesis. Moreover, p*53* is frequently mutated in the early stages of UC-associated carcinogenesis [47] (Fig. 5).

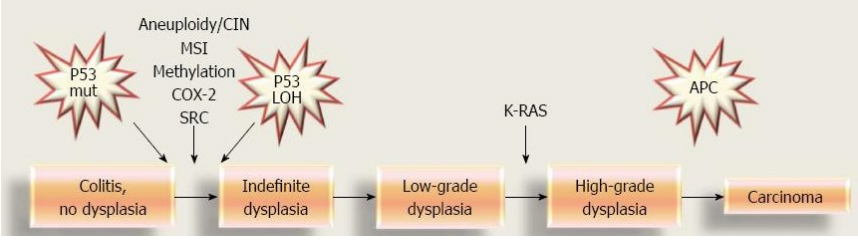


Fig. 5. *Timing of mutation in inflammatory colorectal carcinogenesis [47].*
Chromosomal instability pathway (CIN); microsatellite instability (MSI); p53 loss of heterozygosity (p53 LOH).

Lastly, the sequential pathway of carcinogenesis in UC is influenced by the production of reactive oxygen intermediates (ROIs) due to chronic inflammation. Increased ROIs may cause DNA damage leading to strand breaks and adduct formation [48].

If colonic inflammation is present in Crohn's disease, the risk are reported to be similar to ulcerative colitis [49].

2.2.2 The role of environmental, host and lifestyle factors

Given that ~80% of colorectal cancer cases develop through the accumulation of sporadic mutations, environmental and lifestyle factors are very relevant in colorectal cancer development and progression [50].

Epidemiologic studies have determined a number of risk factors for CRC including age, family history of colon cancer or inflammatory bowel disease, cigarette smoking, diet, race, obesity, physical inactivity and intake of alcohol [34].

Since the 70s, the importance of dietary fibers has emerged, hypothesis supported by other epidemiologic studies in the 80s and confirmed with the one of largest cohort study in the world examining diet and cancer risk (i.e. the European prospective Investigation of Cancer and Nutrition [51–54]), that reported a 40% reduction in colorectal cancer risk between the highest and the lowest quintiles of fibers intake. But interventional studies in which dietary fibers were supplemented to prevent colorectal polyps have failed to demonstrate any difference between treatment and control groups

[55–57] underlying how the role of dietary fibers in colorectal cancer progression is quite complex.

Similarly, to what was said for dietary fibers but with an opposite effect, red meat has a relation with an increased risk of colorectal cancer development. Cooking red meat at high temperature could provoke heterocyclic amines or polycyclic hydrocarbons formations that cold act as potent carcinogens [58]. Additionally, heme iron that is present in red meat could increase cell proliferation and incidence of aberrant crypt foci in colonic mucosa together with an increased faecal concentrations of carcinogens (such as N-nitroso compounds) [59,60].

Dietary fibers and red meat are only two of all the dietary constituents that could have a role in colorectal cancer pathogenesis, in fact, beyond food, other types of factors can influence this process.

Cigarette smoking comprehends a variety of recognized carcinogens (such as polycyclic aromatic compounds, nitrosamines and aromatic amines) and colonic mucosa is certainly exposed to those directly and also via circulation rising the risk of colorectal cancer [61,62]. Heavy alcohol intake is also associated with a high risk of colorectal cancer through its metabolite (e.g. acetaldehyde) and the generation of free radicals [63–66]. But also old age is a risk factor because old age is associated with higher levels of genetic mutations, telomere attrition and DNA methylation changes [67,68].

Furthermore, obesity, high energy intake and sedentary lifestyle have been associated with an increased risk of colorectal cancer [69–71].

2.2.3 Oxidative stress and CRC

Gut mucosa is formed by a layer of epithelial cells and the underlying epithelial connective tissue where are located blood vessels, lymphatics and nerves as well as stromal, inflammatory and immune cells [72]. These components are linked in a complex physiological network to prevent tissue damage and preserve mucosal barrier integrity. Among the major factors causing mucosal damage are gut reactive oxygen species, mainly produced by innate immune cells and mucosa-resident cells [73,74].

Accumulating evidences demonstrated that CRC risk factors like alcohol and smoking were involved in ROS production [75,76] resulting in an altered equilibrium between cellular ROS and antioxidant [77,78]. Uncontrolled and continuous overproduction of free radicals might exacerbate inflammation together with gut barrier disfunction that could lead to enhanced ROS production and inflammation cytokines release [73]. In fact, ROS levels are significantly increased in IBD patients (both with ulcerative colitis and Crohn's disease) and animal models as compared to healthy subjects [79–82]. Similarly, a large body of experimental evidences show that free radicals also extensively take part in development and progression of CRC [74,83–85].

It is widely accepted that ROS induce DNA damages and genetic mutations that are critical causes of CRC [86]. Free radicals cause single and double strand DNA breaks and the common genetic mutations include p53, KRAS, APC and BRAF mutations [76,87]. Furthermore, in recent years several proteins were found as redox sensitive proteins [88], most of which are involved in the initiation and progress of CRC such as Wnt/β-catenin, PI3K/AKT and JAK/STAT signaling pathways [89–91] . Then, the increasing production of ROS could directly regulate the activity of transcriptional factors like NF-κB, p53, HIF-1α and Nrf2 that play a crucial role in colorectal carcinogenesis [92–94].

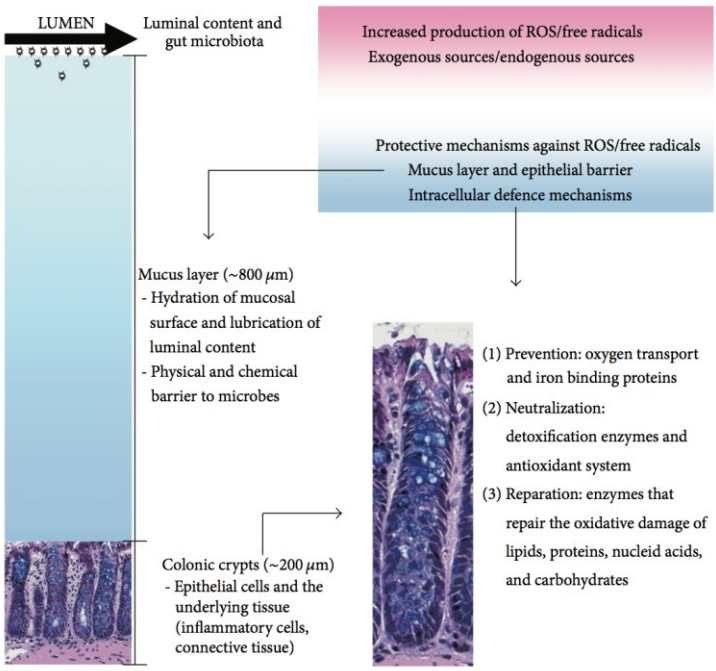


Fig. 6. *Schematic representation of the colonic barrier and intracellular mechanisms against oxidative stress [74].*

2.2.3.1 Reactive oxygen species

Reactive oxygen species (ROS) are products of the regular cellular metabolism that stimulate a variety of signalling pathways both in plant and animal cells responding to intra and extracellular environmental changes [95]. They are regularly generated under normal conditions as a consequence of aerobic respiration [96–98]. Usually, aerobic cells produce superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and organic peroxides. Moreover, under hypoxia the mitochondrial respiratory chain also produces nitric oxide (NO), that can generate reactive nitrogen species (RNS) [97]. Another major source of intracellular ROS is the NADPH oxidases. NADPH oxidases catalyse the production of superoxide from O_2 and NADPH in various tissues in the body; notably the mithocondria have eight known sites that are capable of producing superoxide [99].

Elevated rates of ROS have been detected in almost all cancers, in particular ROS-dependent signaling pathways are persistently elevated in many types of cancers and they can act as second messengers in cellular signaling [99]. Under normal physiological conditions, the intracellular levels of ROS are accurately controlled to prevent cells from damage thanks to detoxification mechanisms. Detoxification from ROS is facilitated by non-enzymatic molecules (i.e. glutathione, flavonoids and vitamins A, C and E) or through antioxidant enzymes which specifically scavenge different kinds of ROS (i.e. superoxide dismutase, catalase and glutathione peroxidase) [100]. When ROS overcome these systems, redox homeostasis is alterated and the result is oxidative stress [96]. Thus, under a continuous environmental stress in which ROS are produced over a long time, a significant damage may occur to cell structure inducing somatic mutations and neoplastic transformation [97].

As previously described, cancer is characterized by a multistage process defined by at least three phases: initiation, promotion and progression; oxidative stress interacts with all three stages of this process. In particular, cancer initiation and progression has been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability and cell proliferation [97]. The beginning of carcinogenesis mediated by ROS may be direct (oxidation, nitration, halogenation of nuclear DNA, RNA, and lipids), or mediated by the different signaling pathways activated by ROS (Fig. 7).

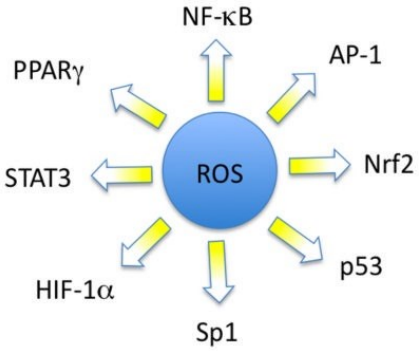


Fig. 7. *Schematic representation of various transcription factors that are modulated by reactive oxygen species* [97].
Peroxisome proliferator-activated receptor gamma (PPARγ); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); Activator protein 1 (AP-1); nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2); Specificity protein 1 (Sp1); Hypoxia-inducible factor 1-alpha (HIF-1α); Signal transducer and activator of transcription 3 (STAT3).

During the progression phase the balancing of ROS generation and ROS scavenging allows cancer cells to remain in the tumorigenic range of ROS levels and tumour cells could express enhanced levels of antioxidant proteins that prevent increased ROS from reaching cytotoxic levels incompatible with growth (Fig. 8) [100,101]. Furthermore, oxidative stress – induced signalling (Fig. 7) events have been described to affect all systems of cancer cell behaviour such as cell survival and apoptosis, morphology, metabolism , motility, adhesion and angiogenesis [92,102,103].

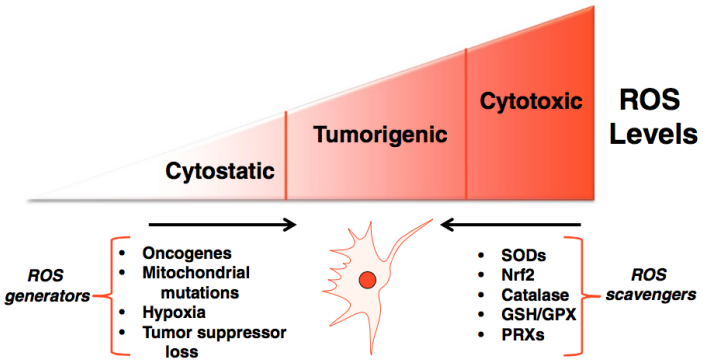


Fig. 8*. Balancing ROS generation and ROS scavenging* [104].
Superoxide dismutase (SOD); nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2); glutathione (GSH); glutathione peroxidase (GPX); peroxiredoxins (PRXs).

Thus, the outcome of direct ROS application or administration of antioxidants in cancer therapy and prevention may be dependent on the tumour type and stage, the type and level of endogenous ROS as well as abundance of ROS – induced survival pathways [99,101]. The induction of oxidative stress can lead to the preferential killing of

cancer cells and various drugs with direct or indirect effects on ROS have been used for effective cancer therapies. Drugs that directly affect ROS metabolism are listed in Table 2 [101].

Table 2. *Classification of anticancer treatments according to their direct or indirect role in regulating ROS levels [101].*

Name	Mechanism of action; effects on ROS	Cancer types	Stage of development
Drugs with a direct role on ROS metabolism			
NOV-002	Glutathione disulphide mimetic; alters intracellular GSSG/GSH ratio	Lung, breast and ovarian cancer	Approved
Sulphasalazine	Inhibitor of cystine/glutamate transporter XCT; reduces intracellular transport of cysteine required for GSH synthesis	Pancreatic and lung cancer	Approved
6-anicotinamide (6-AN)	Inhibitor of glucose-6-phosphate dehydrogenase; reduces GSH	Colon cancer	Approved
L-asparaginase	Depletes glutamine; reduces GSH	Leukaemia, pancreatic cancer	Approved
Small molecule 968 (dibenzophenanthridine)	Glutaminase inhibitor; reduces GSH	Lymphoma, breast cancer	Approved
Buthionine sulphoximine (BSO)	Glutamate–cysteine ligase complex inhibitor; inhibits *de novo* GSH synthesis	Ovarian and breast cancer, melanoma	Approved
Drugs or treatments with an indirect role on ROS metabolism			
Ionizing radiation	Photons (from cobalt, cesium or a linear accelerator) or particles (such as electrons, protons, neutrons, α-particles and β-particles) affect chemical bonds and produce highly reactive free radicals and ROS, which cause damage to DNA and other cellular components	Different types of cancer	FDA-approved
G202	Binds to and blocks the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump, thereby increasing the concentration of cytosolic calcium that causes apoptosis; induction of ROS owing to ER stress	Hepatocellular carcinoma, prostate cancer	Phase II
Celecoxib	Inhibits cyclooxygenase 2 (COX2) activity but it also induces ER stress by causing leakage of calcium from the ER into the cytosol; induction of ROS owing to ER stress	Colorectal cancer, myeloma, Burkitt's lymphoma and prostate cancer	FDA-approved
Nelfinavir	Originally developed as HIV protease inhibitor but it also induces ER stress by an unknown mechanism; induction of ROS owing to ER stress	HPV-transformed cervical carcinoma, head and neck cancer, pancreatic cancer, melanoma and glioma	FDA-approved
N-benzyloxycarbonyl-Ile-Glu (O-t-butyl)-Ala-leucinal (PSI)	Proteasome inhibitor; induces ROS that lead to mitochondrial dysfunction	Leukaemia	FDA-approved
Bortezomib	Proteasome inhibitor; induces ROS owing to ER stress	Mantle cell lymphoma, multiple myeloma	FDA-approved
Anthracyclines (doxorubicin, daunorubicin or epirubicin)	Insert into the DNA of replicating cells and inhibit topoisomerase II, which prevents DNA and RNA synthesis. Induce the generation of oxygen-derived free radicals through two main pathways: a non-enzymatic pathway that utilizes iron, and an enzymatic mechanism that involves the mitochondrial respiratory chain	Different types of cancer	FDA-approved
17-allylaminogeldanamycin (17-AAG)	HSP90 inhibitor; affects protein homeostasis during oxidative stress by disrupting HSP90–client protein complexes and promoting the degradation of the client proteins	Breast cancer, non-small-cell lung cancer	Phase I/II
5-fluorouracil (5-FU)	Inhibits thymidylate synthetase and/or incorporates into RNA and DNA; induces intracellular increase in $O_2^{\bullet-}$ levels	Colon cancer, rectum cancer, and head and neck cancer	FDA-approved
Arsenic trioxide (As_2O_3)	Reacts with cysteine residues on crucial proteins; inhibits mitochondrial respiratory function, thereby increasing free radical generation	Leukaemia, myeloma	FDA-approved
2-methoxyestradiol (2-ME)	Metabolite of estradiol-17β; induces free radicals and loss of mitochondrial membrane potential	Prostate cancer, leukaemia	FDA-approved
N-(4 hydroxyphenyl) retinamide (4-HPR)	Synthetic retinoid derivative; induces apoptosis through the production of ROS and mitochondrial disruption	Prostate cancer, breast cancer, neuroblastoma	FDA-approved
PARP inhibitors	Inhibit the action of the enzyme PARP; reduce the capacity to repair ROS-induced DNA damage	Breast cancer	Phase III
Erastin	Downregulates mitochondrial VDACs and cysteine redox shuttle; alters the mitochondrial membrane permeability and blocks GSH regeneration	RAS^{V12}-expressing tumour cells	Phase I/II
Lanperasone	Downregulates mitochondrial VDACs; alters the mitochondrial membrane permeability	$KRAS^{G12D}$-expressing tumour cells	FDA-approved
AGX-891	Inhibits mutant IDH1 isoform; alters the $NADP^+$/NADPH ratio	Glioma, leukaemia	Preclinical
AG-221	Inhibits mutant IDH2 isoform; alters the $NADP^+$/NADPH ratio	Advanced haematological malignancies	Phase II

2.3 Mouse models of colorectal cancer

There are numerous animal models that approximate some of the characteristics of human CRC, each with its own peculiar advantages and limitations. Thus, any specific experimental issue should be studied by choosing the model best suited to resolve particular tasks [105].

Potential animal models for colorectal cancer fit into four broad categories: spontaneous intestinal cancers, chemically or environmentally induced cancers, cancers induced by genetic manipulation and transplantation models:

1. ***Spontaneous intestinal cancers:*** spontaneous gastrointestinal neoplasia is rare in rodents. A recent study reported that the incidence of intestinal tumours in C57Bl/6J mice fed a common semi-purified diet (AIN-76A, (59)) was 1% in the large intestine and 4% in the small intestine [106];

2. ***Exogenous Promoters of Colorectal Cancer***

   a. *Western Diet-Induced Rodent Neoplasia:* Several rodent studies have been conducted to examine the influence of a diet designed to model a typical "Western diet" on the incidence of colorectal cancer. Several modifications of this diet have been used, but the common features are increased concentrations of fat, with decreased levels of calcium and vitamin D [106,107]. This model is attractive because it appears to capture much of the complexity that underlies spontaneous colorectal carcinogenesis in humans [108]. However, an analysis of the molecular mutations responsible for tumour formation after feeding the Western diet has not been reported. In addition, the dietary level of calcium used is deficient thus, the "Western diet" may change gut physiology in ways that do not reflect the aetiology of human colorectal cancer [108];

   b. *Chemical Induced Models of Colorectal Cancer:* A lot of chemicals are known to have mutagenic potential and many cancer studies have used this characteristic to induce cancer [109]. The most commonly used chemical inducers of colorectal cancer in rodents are the compound 1,2-dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM) [110,111]. The compounds are alkylating agents

that are typically injected intraperitoneally or subcutaneously over several weeks to induce carcinogenesis in the distal colon.

DMH and AOM require metabolic activation to form DNA-reactive products [112,113]. Metabolism of these compounds involves multiple xenobiotic-metabolizing enzymes (Fig. 9), which proceed through several N-oxidation and hydroxylation steps, including the formation of MAM. This is a reactive metabolite that readily yields a methyl diazonium ion, which can alkylate macromolecules in the liver and colon [114,115], including the addition of methyl groups at the O6 or N7 position of guanine (O6-methyl-deoxyguanosine and N7-methyl-deoxyguanosine). The ability of AOM and DMH to target the colonic mucosa is probably a consequence of the relative stability of MAM [116] with a half-life of 12 hours which is sufficient to distribute to the colon [117].

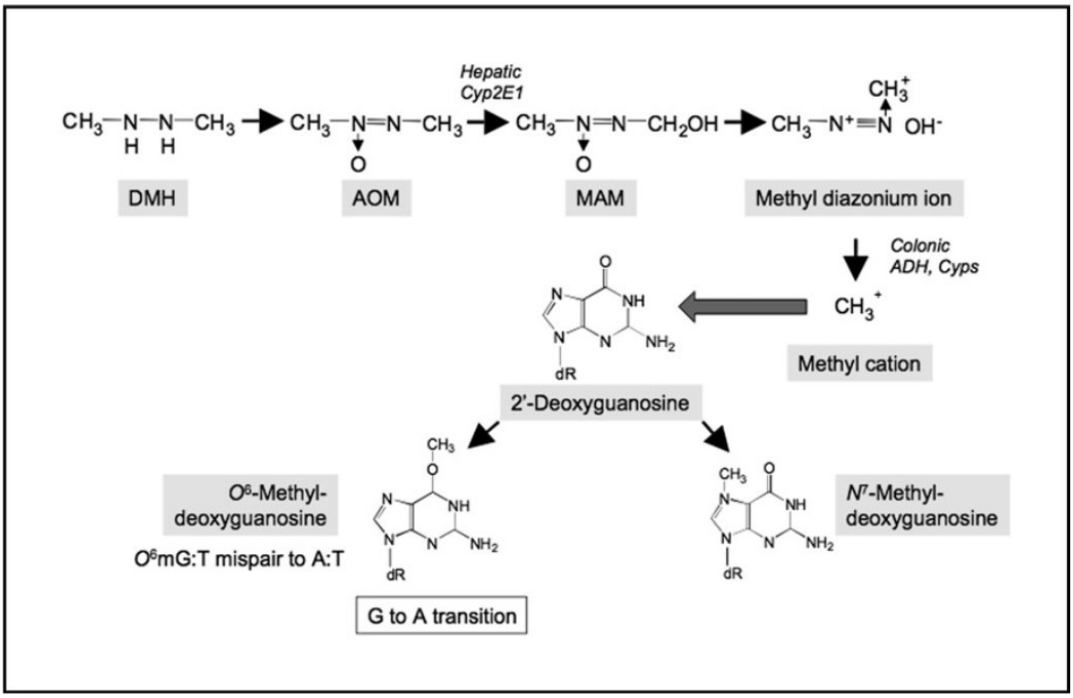


Fig. 9. *Metabolism of DMH and its metabolite AOM [113].*

The majority of these CRC tumours harbour mutations in the β-catenin gene (Ctnnb1), which is similar to HNPCC [118]. In addition, tumour incidence and multiplicity can be altered by both genetic background and by diet [110]. This

makes the models useful for the study of gene-gene and gene-environment interactions that influence the pathogenesis of colorectal cancer [108].

3. ***Mutagen-Induced Germline Mutation Models***: the workhorse for preclinical colorectal cancer research over the past 30 years has been the *Apc*^{Min} mouse. This mouse was identified in 1990 from an ethylnitrosurea (ENU) mutagenesis screen in C57Bl/6J mice. Tumours occur in the small and large intestine, but greater than 10-fold more lesions are found in the small intestine. The genetic basis for the intestinal phenotype is a T-to-A transversion at nucleotide 2549 of the mouse *Apc* gene that truncates the Apc protein at amino acid 850 [119]. Because of its molecular and pathologic similarity to human FAP, *Apc*^{Min} mice have been used extensively to study the development, treatment, and prevention of colorectal cancers that contain somatic *APC* mutations [120,121];

4. ***Genetically Modified Mice***: genetically modified mice offer the potential to precisely recapitulate specific molecular aetiologies relevant to colon cancer by controlling the type, timing, or location of specific genetic alterations. APC, DNA Mismatch Repair genes, β-catenin and KRAS are only few examples of the genes modified to create mouse lines to model human colon cancer (Table 3);

5. ***Transplantation models:*** many models have been developed to monitor the invasiveness and metastasis of the implanted or injected tumours. Nude mice that lack T cell function or SCID mice that lack both B and T cell function have been useful for developing orthotopic tumour implantation models. Grafts from either human (xenografts) or murine (syngeneic autografts or allografts) tumours can be implanted into recipient mice. An important practical limit of orthotopically transplant is the complexity of the surgical procedure involved, along with the lack of immune function that is a necessary element of all xenotransplantation models. These limitations eliminate the principal mechanisms that govern the effects of inflammation and immunity on tumour development [36,108,122].

Table 3. *Selection of genetically modified mouse models of CRC* [36].

Mouse allele	Rationale	Strategy	Advantages	Disadvantages
Apc$^{Min/+}$ [47, 48]	• N/A • Ethylnitrosurea (ENU) mutagenesis screen • Tumour multiplicity easily quantifiable	• Germline truncating mutation (N terminus) • Loss of heterozygosity occurs at wildtype allele in tumours	• Model of human familial adenomatous polyposis (FAP) syndrome and >80% of sporadic CRCs contain mutations [2, 4] • Short-lived for rapid studies	• Developed hundreds of low-grade adenomas • Short-lived as tumour burden causes obstruction, prolapse and bleeding • Short life-span means that adenomas do not acquire sufficient mutations to progress to adenocarcinoma and metastasise [56, 57] • Tumours predominantly located in small rather than large intestine
Msh2$^{-/-}$ [54]	• *Msh2* mutations common in CRC	• Germline knock-out	• Model of Hereditary non-polyposis CRC (HNPCC) or Lynch Syndrome (3% of all CRCs)	• *Msh2* mutation in all cells of body and mice are predisposed to lymphomas
Villin-Cre/ Msh2^{LoxP} [55]	• To restrict malignancy to intestine to prevent lymphoma	• Conditional • *Cre* recombinase[a] expressed from promoter of intestinal specific gene (*Villin*)	• Developed intestinal adenomas and adenocarcinomas • No deaths from lymphoma	• Do not develop metastases
Apc$^{580S/580S}$ [64]	• To model advanced CRC by restricting tumours to colon and reducing tumour burden	• Conditional Adenoviral Cre recombinase[a] is administered through the anus to inactivate *Apc*	• Live >1 year. • Developed two or three intestinal adenomas • Some mice developed adenocarcinomas	• Do not develop metastases • Conditional allele (unactivated) reduces *Apc* expression in all cells, similar to *Apc*$^{fl/fl}$ [10], which results in development of life-limiting hepatocellular carcinomas by 14 months (REM unpublished observations of *Apc*$^{580S/580S}$)
Apc$^{lox15/+}$; *Fabpl-Cre* [109]	• To model advanced CRC by restricting tumours to the colon and reducing tumour burden	• Conditional • *Cre*-recombinase[a] is expressed in the distal small intestinal and colonic epithelia to inactivate *Apc*	• Live >1 year. • Developed two or three intestinal adenomas • Some mice developed adenocarcinomas	• Do not develop metastases
Apc$^{Min/+}$*Trp53*$^{-/-}$ [110]	• To model advanced CRC through addition of cooperating gene mutations	• Germline mutations in *Apc* and *Trp53*		• Do not develop metastases
Apc$^{2lox14/+}$ *KrasLSL-G12D* and *Fabpl-Cre* [111]	• To model advanced CRC through addition of cooperating gene mutations	• Conditional • *Cre*-recombinase[a] is expressed in the distal small intestine/colonic epithelia and results in constitutive activation of K-Ras and inactivation of Apc	• Developed more adenocarcinomas than single mutant (Apc or Kras alone) control mice	• Do not develop metastases
Apc mutant with disruption of *Tgfbr2*, *Smad2* or *Smad4*. [112–114]	• To model advanced CRC through addition of cooperating gene mutations		• Developed more adenocarcinomas than single mutant control mice.	• Do not develop metastases
Apc$^{Min/+}$ *Villin Cre Fbxw7*$^{(\Delta G)}$ [115]	• To model advanced CRC through addition of cooperating gene mutations	• Germline mutation of *Apc* • Conditional mutation of Fbxw7 • Cre recombinase[a] expressed from promoter of intestinal specific gene (*Villin*)	• Decreased lifespan • Increased tumour burden • Fbxw7 null control mice developed adenomas by 9-10 months of age	• Do not develop adenocarcinomas or metastases
Apc$^{CKO/CKO}$-*LSL*-G12D; *Kras*$^{tm4tyj/+}$ [62]	• To model advanced CRC through addition of cooperating mutations. To restrict tumours to the colon and reduce tumour burden	• Adenoviral *Cre* solution administered via the anus to simultaneously disrupt *Apc* and activate K-ras	• Developed adenocarcinomas after five months • Developed metastases to distant organs after six months e.g. liver • In vivo monitoring via colonoscopy	• Labour intensive mouse surgery required to clamp a section of colon to deliver Adenoviral *Cre* solution to the colon via the anus

CRC, Colorectal carcinoma.

[a]*Cre* recombinases catalyse recombination between two *loxP* sites that are situated in the introns surrounding a critical exon(s) and result in excision of the DNA between the sites.

Table 4. *Transplantation model of CRC* [36].

Transplantation model	Strengths	Weaknesses
Subcutaneous CRC cell line xenograft: injection of cells e.g. HCT116 and HT29 to immune deficient mouse	• Low cost • Rapid tumour growth (2 weeks) • Well characterized cell-lines: gene mutation status, transcriptome and drug response data available [73, 106, 107] • Easy to genetically manipulate prior to transplantation e.g. with inducible shRNA or by CRISPR/Cas9 [108] • Model accessible to the majority of research labs	• Representative of advanced disease • Have undergone significant clonal selection [73] • Microenvironmental differences (cells derived from colorectum injected under skin) • Species mismatch in tumour (human) and stromal (mouse) cell may limit cross-talk • Immune deficient host • Rarely metastasise
Orthotopic xenografts of CRC cell lines: injection of cells into intestinal serosa of immune deficient mouse	• As above • More natural microenvironment for CRC cells • Some metastasise to liver e.g. from HCT116 or HT29	• As above (except for 'rarely metastasise') • May require surgery to implant cells
Patient-derived xenografts (PDXs): Suturing of 1–2 mm fragments of fresh surgical specimens of CRC, to intestine of immune deficient mouse	• Reproducible liver metastasis • Avoid selection of dominant clones during long-term cell culture • Temporary preservation of species-specific tumour-stromal cell cross talk • More natural microenvironment for CRC cells	• Not readily scalable • Host (mouse) stromal cells replace human stromal cells within a few weeks (species mismatch) • Immune deficient host • Limited by availability of surgical specimens • Expensive (labour intensive and time consuming)
Syngraft/Isograft: Suturing 1–2 mm mouse tumour fragments or mouse tumour cell lines e.g. MC38 cells to a genetically identical inbred, immune competent mouse	• No species mismatch between tumour and stromal cells • Host has intact immune system that enables testing of immunomodulatory anti-cancer agents	• Expensive (labour intensive and time consuming) • The model is not human

2.4 The immune system

As previously described it is known that host factors have an important role in both cancer progression and patient survival. In addition, advances in molecular biology and immunology have demonstrated that CRC is immunogenic and the presence and activation status of immune cells in the tumour microenvironment have great prognostic implications [63,123–125].

The immune system has two lines of defence that are called innate and adaptive immunity. Innate immunity is the first, non-specific (antigen-independent) mechanism of defence and it has no immunological memory. On the contrary adaptive immunity is antigen-dependent, antigen-specific and generate memory cells [126,127].

Through the production of cytokines, the primary function of innate immunity is the recruitment and activation of immune cells. Different cellular populations are involved in these responses such as macrophages and dendritic cells (DCs), with a role in phagocytosis and in antigen presentation to T cells, and natural killer (NK) cells that play an important role in the rejection of tumours and destruction of cells infected by viruses [126,127].

The main functions of the adaptive immune response are the recognition of specific “non-self” antigens in the presence of “self” antigens that lead to pathogen-specific immunologic effector pathways in order to eliminate definite pathogens or pathogen-infected cells. Moreover, the adaptive immune response develops an immunologic memory that can quickly eliminate a specific pathogen. Cells of the adaptive immune system are: the effectors of cellular immune responses the T lymphocytes, which mature in the thymus, and antibody-producing cells, the B lymphocytes, which arise in the bone marrow [128–130].

T-cells and APCs express the T-cell receptor (TCR) to recognize a specific antigen and the surface of APCs express cell-surface proteins called major histocompatibility complex (MHC). MHC are divided in class I molecules (also known human leukocyte antigen HLA A, B and C) that present endogenous peptides and class II (also termed HLA, DP, DQ and DR) that present exogenous peptides. T cells are activated when they meet an APC which has internalized an antigen and is expressing antigen fragments bound to its MHC molecules. This specific interaction the TCR and the T cells secrete cytokines that will regulate the development of the immune response [126] (Fig. 10).

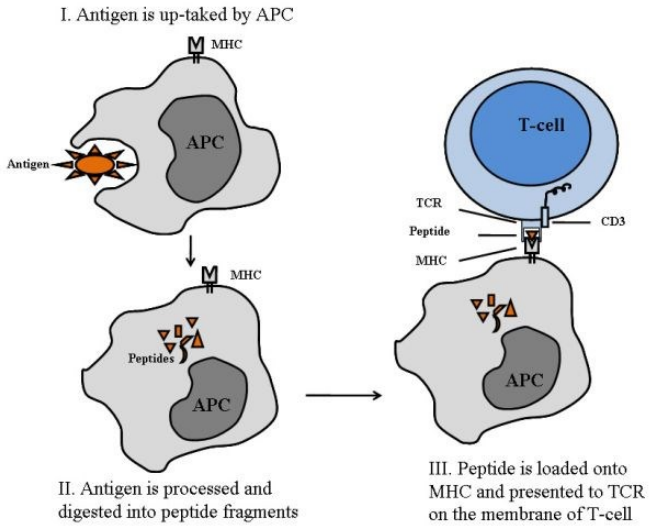


Fig. 10. *The induction of T-cell response [131].*
This activation depends on two combined signals provided by antigen-presenting cells. The first one is interaction of T-cell receptors with antigen on the major histocompatibility complex. Antigen presenting cell (APC); T cell receptor (TCR); major histocompatibility complex (MHC).

The antigen presentation process stimulates T cells to differentiate into cytotoxic T cells (CD8+), that are activated by MHC class I molecules, or T helper (Th) cells (CD4+),

activated by MHC II, that contribute to optimize the further immune responses. An additional type of T cell is the regulatory T cell (T reg), that limits the amplitude of immune responses and suppresses the immune system [126].

2.4.1 Co stimulatory molecules: the B7 family

Antitumor immune response is mainly enhanced by CD8+ cytotoxic T lymphocytes and CD4+ helper T lymphocytes [132]. In particular in antitumor immune response, CD8+ T cells recognize peptides (usually 8-10 amino acids) derived from TAAs bound by MHC class I molecules on tumour cells. In contrast, CD4+ T cells recognize peptides (usually 10-30 amino acids) in association with MHC II molecules on APCs and enhance the persistence on antigen-specific CD8+ CTLs [133]. The induction of T-cell responses depends on two combined signals provided by antigen-presenting cells. The first one is interaction of T-cell receptors with antigen on the major histocompatibility complex, and the second is delivered by co-stimulatory molecules through their counter-receptors (CD28) on the T lymphocytes. In the absence of co-stimulation, T-cell receptor antigen interactions induce an anergic state in T cells (Fig. 11) [132,134,135].

The family of B7 co-stimulatory molecules consists of at least two members, B7.1 (CD80) and B7.2 (CD86). B7.1 and B7.2 are members of the Ig supergene family and are expressed by hemopoietic cells, including monocytes/macrophages, B cells, dendritic cells, and T cells. Both molecules can be induced after stimulation with a number of stimuli, such as LPS and IFN-γ [136].

Research suggests that CD86 mediates the initiation phase of T cell responses. In contrast, CD80 contributes more towards maintenance through CD28 receptor activation or termination of the ongoing T-cell response through cytotoxic T lymphocyte antigen (CTLA)-4 (CD152) signalling [137,138].

After recognition of MHC peptide complex by the TCR, the second signal required for T cell activation is provided by binding of CD80 or 86 to CD28 on the T cells. This interaction drives to cell surface the expression of CTLA-4, that has a higher affinity for CD80/86, thus stopping the activation signal. Moreover, the CTLA-4 signal down-regulates T cell function and inhibits excessive expansion of activated T cells. Anti-CTLA-

4 monoclonal antibodies bind to CTLA-4, and block the interaction with CD28 recetor, that is again free to interact with CD80/86, extending T cell activation and increasing T cell-mediated immunity against tumours [139].

Interestingly, in CRC, CD80 expression in mouse tumour cells of epithelial origin has been confirmed. Accordingly, it should not be expected that CD80 would function differently on the colon cancer cells compared with professional antigen presenting cells [140].

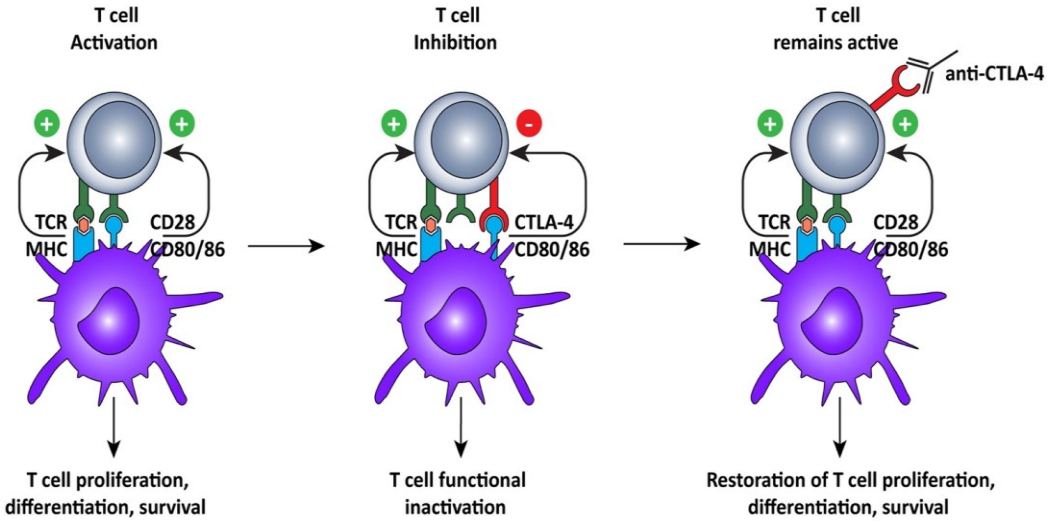


Fig. *11*. *Cytotoxic T lymphocyte-associated antigen-4 blockade restores T cell activation [139]. T lymphocyte receptor (TCR); major histocompatibility complex (MHC).*

2.4.2 The role of the immune system in the carcinogenesis

Lewis Thomas and Frank Macfarlane were the first to formally propose the hypothesis called *cancer immunesurveillance* telling that the immune system is able to prevent cancer formation in immunocompetent hosts by recognizing and killing tumour cells [141].

It is now proposed that cancer immunosurveillance it's just a part of a more complex process called *immunoediting* (Fig. 12) where immune cells also differentiate tumour immunogenicity. In fact, the immune system plays different roles in preventing cancer: it protects host from viral infection that could promote tumour, it prevents the development of an inflammatory environment that facilitates tumorigenesis and it also eliminates tumour cells. However, recent studies have evidenced that the immune system can also promote tumour progression by selecting for tumour cells that are more

fit to survive in an immunocompetent host or by establishing conditions within the tumour microenvironment that facilitate tumour outgrowth [142,143].

In particular, cancer immunoediting consists of three steps:

1. Elimination, the innate and adaptive immune systems work together to detect the presence of a transformed cells and destroy it before it before it generates a tumour clinically apparent [142]. During the elimination phase immune effector cells such as cytotoxic T cells (CTL's) and NK cells with the help of dendritic and CD4+ T cells are able to recognize and eliminate tumour cells. This killing relies on stress ligands such as NKG2D and recognition of TAA's (tumour associated antigens) in the TCR-MHC complex [144].
2. Equilibrium. A sporadic cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its growth is prevented by the immune system. T cells, IL-12, and IFN-γ are essential to maintain tumour cells in a state of functional latency, while NK cells and molecules that participate in the recognition or effector function of cells of innate immunity are not necessary; this indicates that equilibrium is a function of adaptive immunity only [142,145];
3. Escape. In the escape phase, tumour cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as progressively growing, visible tumours. Progression from equilibrium to the escape phase can occur because the tumour cell population changes in response to the immune system's editing functions and/or because the host immune system changes in response to improved cancer-induced immunosuppression or immune system decline [142]. Tumour cells can secrete cytokines that recruit suppressive cells such as Treg cells, immature myeloid cells (comprising immature dendritic cells (iDC) and myeloid-derived suppressor cells (MDSC)), and M2 macrophages. iDC might cause T-cell anergy due to absence of co-stimulatory molecules expression. M2 macrophages and MDSC inhibit T-cell responses through a variety of mechanisms, including nutrient sequestration via arginase, ROS generation, nitric oxide (NO), as well as interference with trafficking into the tumour site. Immunosuppressive cytokines and the up-regulation of immunosuppressive

enzymes (like indolamine-2,3-dioxygenase (IDO) and arginase) that catabolize essential nutrients essential for effector cell activation and also generate immunosuppressive catabolites, contribute to generate a microenvironment where immune responses are difficult to originate and to be sustained. Additionally tumour cells will loose expression of antigenic molecules, down-regulate MHC molecules, and up-regulate inhibitory molecules such as PD-L1 [144].

Loss of tumour antigen expression is one of the best-studied escape mechanisms, and it can occur in at least three ways: through emergence of tumour cells that lack expression of strong antigens, through loss of major histocompatibility complex (MHC) class I proteins that present these antigens to tumour-specific T cells, or through loss of antigen processing function within the tumour cells that is needed to produce and load the antigenic peptide epitope and onto the MHC class I molecule [142]. Furthermore, another well recognized escape mechanism consists in the down-regulation of costimulatory molecules: indeed, many tumours lack the expression of positive costimulatory molecules such as CD80 and CD86 but retain the ability to present antigens on MHC I, therefore leading to T cell anergy rather than immune cells activation [146].

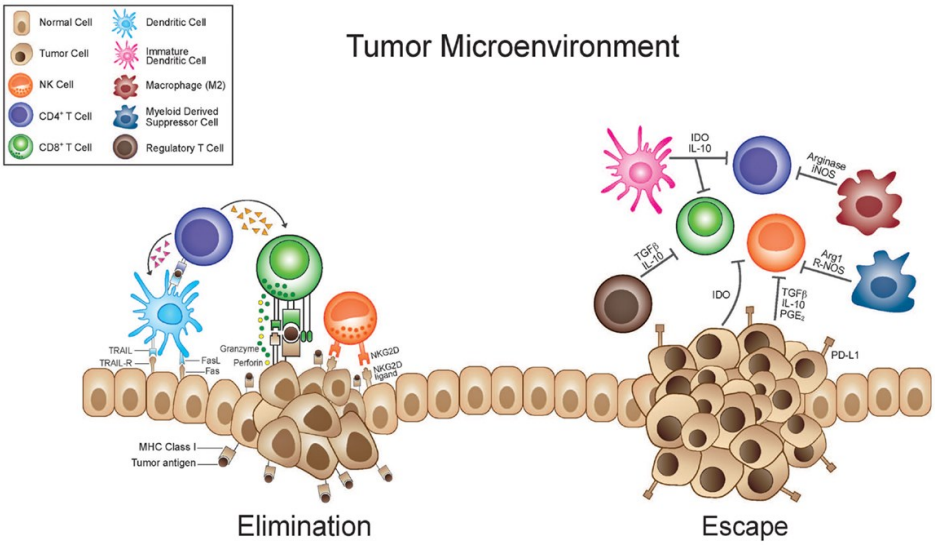


Fig. 12. *Immunosuppressive tumour microenvironment and antigen loss mediate tumour escape* [144].

Cytotoxic T cells (CTL's); Natural Killer (NK) cells; T-helper lymphocyte (CD4+); cytotoxic lymphocyte (CD8+); Natural Killer Group 2D (NKG2D) receptor; tumor associated antigens (TAA's); regulatory T (Treg) cells; immature dendritic cells (iDC); myeloid-derived suppressor cells (MDSC); reactive oxygen species (ROS) generation; nitric oxide (NO); indolamine-2,3-dioxygenase

(IDO); inducible nitric oxide synthase (iNOS); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); Transforming growth factor beta (TGFβ); interleukin 10 (IL-10); prostaglandin E2 (PGE_2).

2.4.2.1 The Cancer-Immunity Cycle and the immune checkpoints in CRC

In order to fight and eliminate cancer cells a series of different steps must be started and propagate in the host. Dan Chen and Ira Mellman developed the *cancer immunity cycle* that has become the intellectual background for cancer immunotherapy research (Fig. 13). In the first stage of this cycle, DCs capture and process neoantigens created by oncogenesis for the next presentation on MHC I and MHC II molecules to T cells (step 2) in order to activate T cell responses against the cancer – specific antigens (step 3). Then, the activated T cells infiltrate the tumour (step 4 and 5) and specifically recognize cancer cells through their TCR and its related antigen bound to MHC I (step 6) in order to kill their target cancer cells (step 7). This final process releases more tumour-associated antigens (step 1 again) to increase the width and intensity of the response. However, in cancer patients, the Cancer- Immunity Cycle does not generally perform optimally [147]. At the moment multiple hypothesis have been proposed: tumour antigens may not be revealed, DCs and T cells may treat antigens as self rather than non – self so generating T regulatory cell responses instead of effector responses, T cells may not properly direct to tumours, may be inhibited from infiltrating the tumour, or factors in the tumour microenvironment might inhibit those effector cells that are produced [148].

The relevance of anti-cancer immune response to control tumour growth is outlined by the introduction of cancer immunotherapy in several protocols. Cancer immunotherapy has the aim to initiate or reinitiate a self – sustaining cycle of cancer immunity, allowing it to increase and propagate, but not so much as to uncontrolled autoimmune inflammatory responses. The most effective tactics include selectively targeting the rate-limiting step and a common rate – limiting stage is the *immunostat function*, immunosuppression that occurs in the tumour microenvironment [149,150]. Thus, the clinical application of antibodies that block immune checkpoints has become very important in order to restore T cells functions in normal physiologic settings, that routinely lead to tumour elimination [151].

The FDA has so far approved immune checkpoint inhibitors to treat certain tumours such as melanomas, lung cancers, and kidney cancers [152]. The main targets of these new immunotherapeutic are PD-1 and its ligands. Programmed death-1 (PD1) is a coinhibitory receptor that is an inducible molecule expressed on CD4+ T cells, CD8+ T cells, NKT cells, B cells and monocytes/macrophages. PDL-1 (constitutively expressed on a wide variety of immune and non-immune cells) and PDL-2 (its expression is dependent on microenvironment) are known ligands of PD1 [153]. By preventing the PD-1 protein from engaging PD-L1, which is expressed mainly by tumour cells, these immunotherapeutic suppress the immune-inhibiting signals transmitted to T cells (Fig. 14).

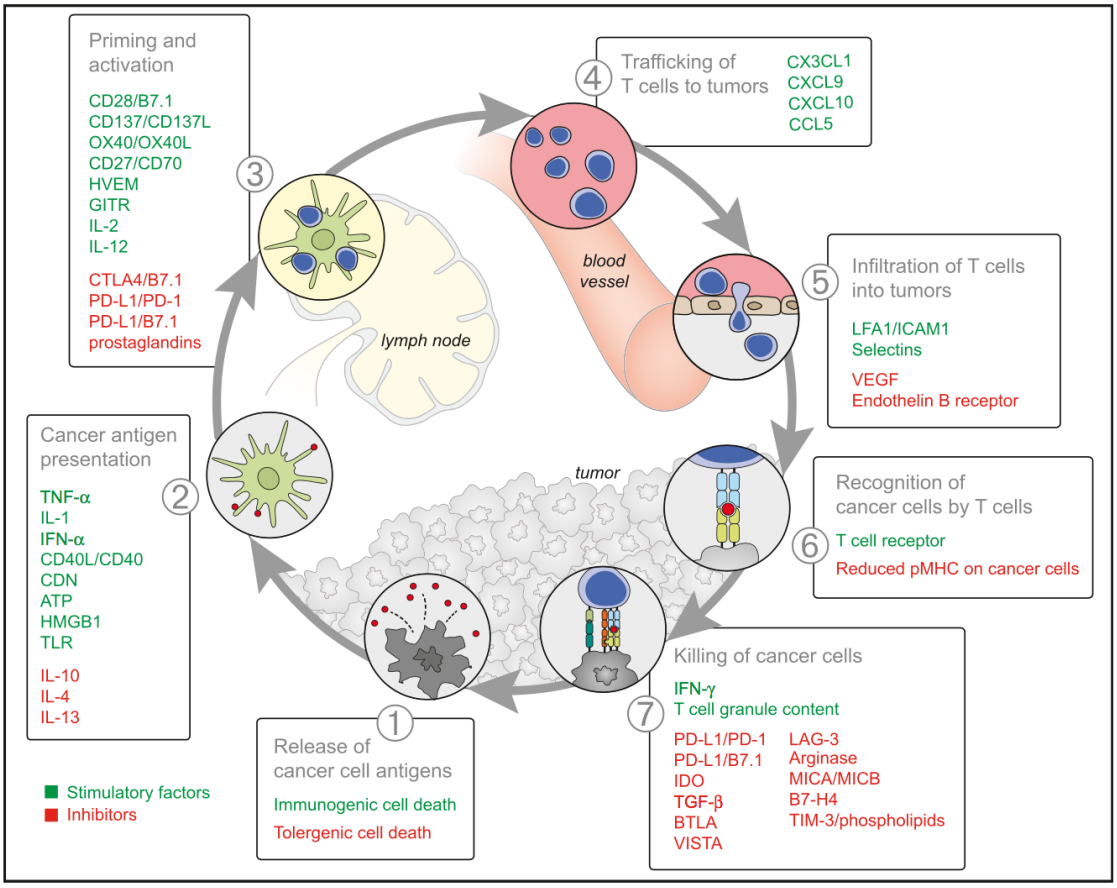


Fig. 13. *The cancer immunity cycle by Dan Chen and Ira Mellman [147].*
Each step of the Cancer-Immunity Cycle requires the coordination of numerous factors, both stimulatory and inhibitory in nature. Stimulatory factors shown in green promote immunity, whereas inhibitors shown in red help keep the process in check and reduce immune activity and/or prevent autoimmunity. Immune checkpoint proteins, such as CTLA4, can inhibit the development of an active immune response by acting primarily at the level of T cell development and proliferation (step 3). We distinguish these from immune rheostat (''immunostat'') factors, such as PD-L1, can have an inhibitory function that primarily acts to modulate active immune

responses in the tumor bed (step 7). Examples of such factors and the primary steps at which they can act are shown. Abbreviations are as follows: IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CDN, cyclic dinucleotide; ATP, adenosine triphosphate; HMGB1, high-mobility group protein B1; TLR, Toll-like receptor; HVEM, herpes virus entry mediator; GITR, glucocorticoid-induced TNFR family-related gene; CTLA4, cytotoxic T-lymphocyte antigen-4; PD-L1, programmed death-ligand 1; CXCL/CCL, chemokine motif ligands; LFA1, lymphocyte function-associated antigen-1; ICAM1, intracellular adhesion molecule 1; VEGF, vascular endothelial growth factor; IDO, indoleamine 2,3-dioxygenase; TGF, transforming growth factor; BTLA, B- and T-lymphocyte attenuator; VISTA, V-domain Ig suppressor of T cell activation; LAG-3, lymphocyte-activation gene 3 protein; MIC, MHC class I polypeptide-related sequence protein; TIM-3, T cell immunoglobulin domain and mucin domain-3.

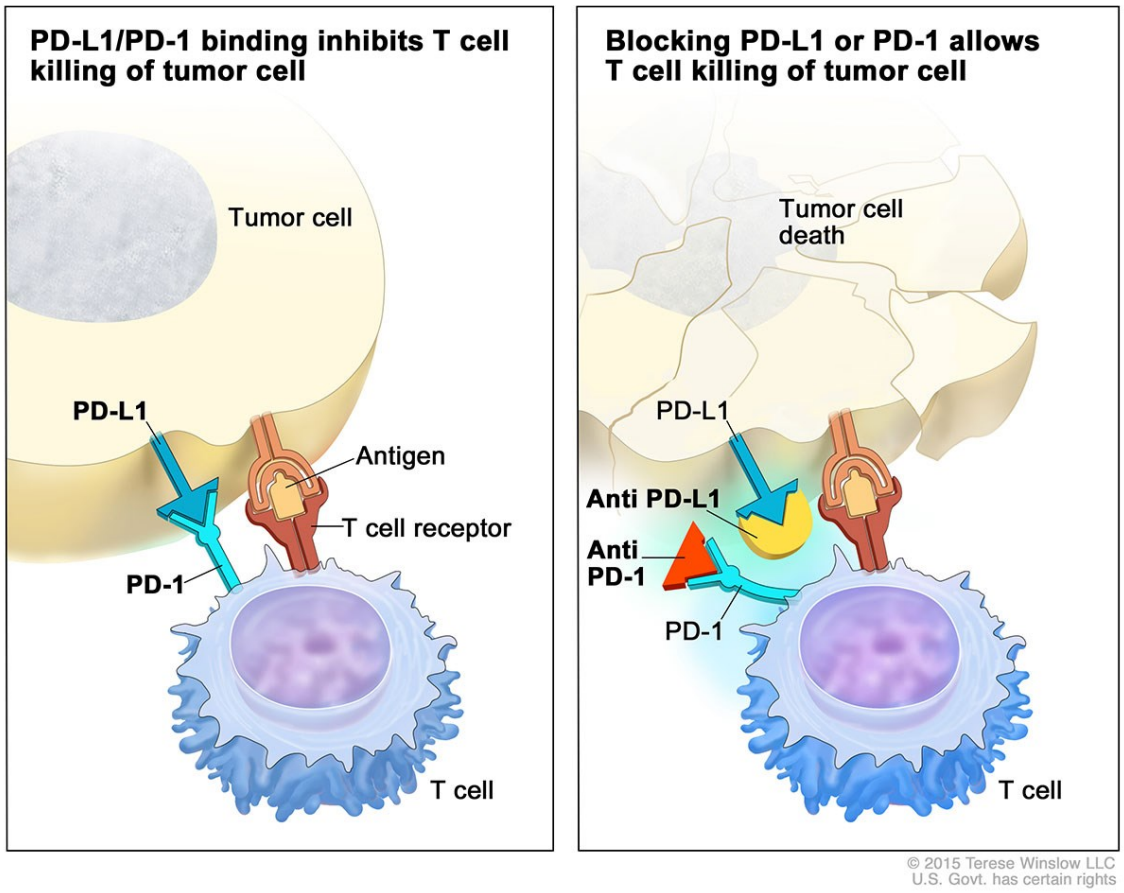


Fig. 14. *PD-L1/PD1 binding.*
Credit: National Cancer Institute/Terese Winslow.

As regards colorectal cancer immunotherapy, it seems that immune checkpoint blockade could be more effective in microsatellite instability (MSI) colorectal cancers, probably because MSI tumours are highly infiltrated with activated CD8-positive cytotoxic T cell lymphocytes [152,153]. In fact, microsatellites have a central biological importance: they cause DNA polymerase slips in the replicative fork causing DNA mismatches and ultimately protein mutations creating source of mutations zones and

commonly frameshift resulting in large changes in proteins that are extremely immunogenic [154].

Moreover, it was also demonstrated that cytotoxic T lymphocyte antigen-4 (CTLA-4), another molecule involved in T lymphocytes inhibition and possible target of immunotherapeutic, is expressed at considerably higher levels in MSI tumours [153]. Very recently, the FDA has approved the combination use of Nivolumab (that blocks PD-1) and Ipilimumab (anti CTLA-4) for patients with previously treated MSI-H (microsatellite instability high) and DNA mismatch repair deficient (dMMR) metastatic colorectal cancer [155].

With the aim to upgrade the immunological status of CMS2 and CMS4 groups that are poorly immunogenic given the absence of MSI, the novel CEA-TCB, the first T cell bispecific antibody with a novel 2-to-1 format that has been optimized for efficacy and safety very recently, might be a new therapeutic approach to treat CRC [156,157].

This bispecific antibody binds simultaneously with one arm to CD3 on T cells and with the second arm to CEA on tumour cells. Thus, carcinoembryonic antigen (CEA) is a 180 kDa glycoprotein broadly known as a common tumor biomarker that is over-expressed in various solid tumor including colorectal, pancreatic, gastric, non-small cell lung, breast, and other cancers. Moreover, the inert engineered Fc region gives to the antibody a significantly longer circulatory half-life allowing for systemic administration every few weeks and reduces the risk of adverse infusion reactions [156,157]. Overall, CEA TCB treatment inhibits tumor growth and generates a highly inflamed tumor microenvironment.

2.4.3 Mechanisms regulating CD80 expression

CD80 is programmed for expression by professional APC, which include macrophages, dendritic cells, Langerhans' cells, and B cells. On the other hand, non-professional APC usually do not express CD80. However, recent experimental studies published by our group and others have challenged this view, reporting that epithelial cells in some tissues can express CD80 [43,140,158].

So far studies on CD80 expression focused on its regulation mainly in professional APC. CD80 mRNA is detectable in tuberculin-purified protein derivative (PPD) stimulated normal or leukemic B cells within 4 to 6 h, and peaks at 16 to 18 h. The CD80 protein on the cell surface is revealed by 16 h, and peaks approximately at 72 h after stimulation. Thus, the expression of the CD80 gene appears to be tightly regulated in B cells [159]. In contrast, other APCs (i. e. macrophages and especially dendritic cells) can express low levels of CD80 constitutively and can be readily induced to express higher levels of this accessory molecule by certain cytokines and/or by CD40-cross-linking. T cells, on the other hand, express CD80 only after long-term culture with IL-7, or following repeated stimulation [160]. A study focused on dendritic cells, showed that low oxygen tension (hypoxia) inhibits expression of several differentiation and maturation markers, including CD80, in response to lipopolysaccharides [161]. Another one, demonstrated that hypoxia selectively reduce the surface expression of the CD80 on human monocyte, a reduction confirmed by using the mouse macrophage cell line RAW 264.7 [162].

It has been also shown that exposure of P815 tumour cells to melphalan (L-phenylalanine mustard; L-PAM) leads to upregulation of CD80 surface expression, and this L-PAM-induced upregulation requires RNA synthesis and is associated with accumulation of CD80 mRNA. The effect of L-PAM on CD80 surface expression can be mimicked by exposing P815 tumour cells to oxidative stress. Moreover, the antioxidant *N*-acetyl-L-cysteine prevented the L-PAM- induced accumulation of CD80 mRNA in P815 tumour cells, suggesting that reactive oxygen species are involved in the transcriptional regulation of L-PAM-induced B7-1 gene expression through NFkB [163].

Indeed, Scarpa et al. observed significant correlation between oxidative DNA damage and CD80 mRNA and protein levels in the colonic mucosa along the inflammatory oncogenic pathway [48].

As such, the molecular mechanisms that activate or repress and control the tissue specificity of CD80 transcription are likely to be complex. Despite the importance of CD80, the critical transcription factor(s) for CD80 gene expression is still unknown. Transcription factors involved in stimulus-induced expression of CD80 such as interferon regulatory factor 7 (IRF7) and nuclear factor κ-light-chain-enhancer of activated B cells

(NFkB) have been observed in some promoter structure analyses. However, the specific transcription factors regulating the basic and constitutive expression of CD80 have not been identified to date [164].

2.4.4 CD80 molecule in CRC progression

Given that the initial phases of carcinogenesis are crucial in order to fight the tumor onset, it could be fundamental to enhance the immune system before the escape mechanisms establishment. According to this concept, in a previous work Scarpa et al. showed that CD80 expression was increased in epithelial cells from dysplastic colon tissue of UC patients but not in neoplastic lesions, suggesting that IECs act in the initial stages of carcinogenesis as APCs. Moreover, in an animal model of CRC associated to colitis, they underlined that CD80-CD28 signalling controls the progression from low grade dysplasia (LGD) to high grade dysplasia (HGD), confirming the role of IECs in the immunosurveillance mechanisms acting in the early stages of carcinogenesis. The dysregulation of this primary immune checkpoint may allow the progression from LGD to HGD and invasive cancer [165].

Consistent to this view, we observed a significant increase of CD80 expression in epithelial cells of preneoplastic lesions compared to control tissues (Fig. 15A), along with a significantly increased in percentage of CK+ HLA ABC+ in adenoma as compared to control and tumoral mucosa (Fig. 15B). Moreover, by exploring the NCBI-GEO database, we analysed an independent study on laser microdissected human CEC [166]. The microarray data set presented a significant up-regulation of CD80 expression in adenoma derived CEC vs normal mucosa derived CEC as well as vs carcinoma derived CEC (Fig. 15C).

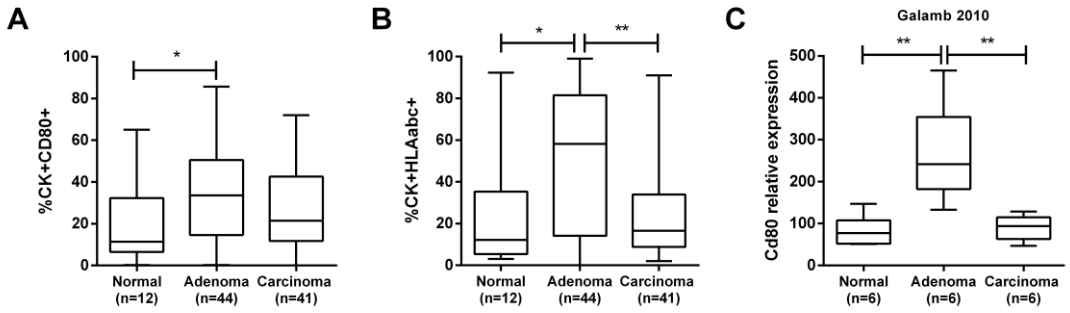


Fig. 15. *CD80 is overexpressed by epithelial cells in human colon preneoplastic lesions*
A and B) Human dysplastic colonic mucosa and healthy mucosa specimens were analysed by flow cytometry for CK+CD80 and CK+HLA Abc+. C) CD80 relative expression in adenoma derived CEC vs normal mucosa derived CEC as well as vs carcinoma derived CEC, from Galamb et al. 2010.

Consequently, the increased CD80 expression and antigen presenting activity by colonic epithelial cells appears limited to pre-cancerous lesions, underlying how CD80 expression on dysplastic epithelial cells is involved in the immune surveillance mechanisms of sporadic CRC.

Interestingly, in even more advanced neoplastic stages, when the tumor acquires the ability of invading the surrounding tissues, it has also reported a reactivation of the immune system. This phenomenon includes cd4+, cd8+ and cd68+ cells accumulation on the invasive front of the tumor mass, compared to the intratumoral zone [167]. Similarly, in the cases in which metastatic cells accumulated inside lymph nodes, they lead to invade the extracapsular area. This feature, called extra-nodal extention (ENE), shows a similar immune system cells concentration described above and, in particular, it is characterized by a marked increase in CD80 expression [167].

2.5 The gut microbiome

In recent years, inflammation within the intestine in addition to diet, lifestyle, has been related to the gut microbiota [168].

The human gastrointestinal tract is colonized immediately after birth, initially by a limited number of microbes that with the age reach more than 10^{14} microbes including more than 1000 different species of bacteria, viruses, archaea, and fungi [169,170] with Firmicutes and Bacteroidetes as dominant phyla [171–173]. Moreover, the gut microbiota

has to be considered as a dynamic ecosystem evolving during the life as a result of genetic, diet, drugs and antibiotics, environment, and lifestyle factors (Fig. 16) [174].

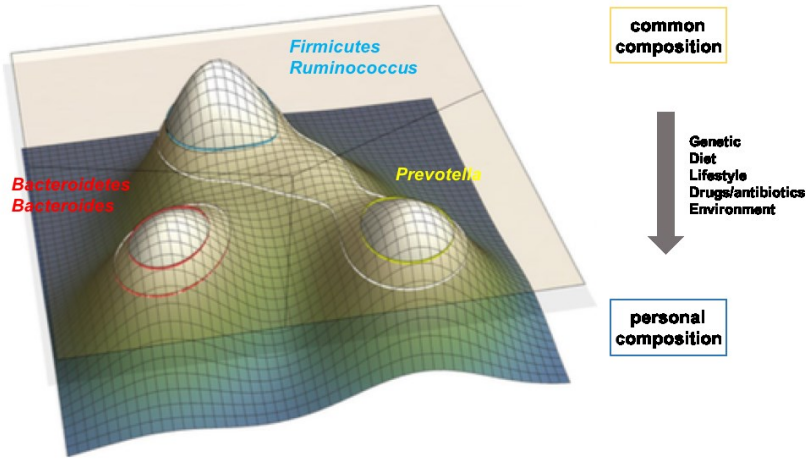


Fig. 16. *Schematic representation of the microbial composition landscape [174]. Adapted from Costea PI, et al. Nature Microbiology 2018;3:8.*

Symbiotic members of gut microbiome are often commensal, signifying that they benefit from the relationship with the host without negative effects. Moreover, this relationship could be considered as symbiotic, meaning that an intact microbial community is fundamental for host development and health [175,176]. In fact, in addition to digestion function, the gut microbiome has an important role in the proper development and maturation of the immune system. Thus, Germ-free mice, that are lacking microbes, have an underdeveloped immune system and inefficient mucosal barrier [177,178].

Colorectal tumour promoting effects of the microbiota seem to be caused by altered host microbiota interactions and by *dysbiosis*, defined as an abnormal community structure or composition of the gut microbiome [179–185] given by various environmental factors, including the use of antibiotics, lifestyle, diet and hygiene preferences [186]. Several studies have shown that dysbiosis can be observed in CRC patients faeces compared to healthy controls [187–192].

Another important connection between the microbiota and CRC is inflammation. Dysbiosis causes an increased level of endotoxin and oxidative stress with an impaired detoxification system activity leading to a systemic inflammation. Moreover, the gut microbiota has a profound influence on the homeostasis in the intestinal immune system both initiating inflammation and regulating immune system [168]. During

homeostasis, the gut microbiota has important roles in the development of intestinal immunity but dysbiosis results in a loss of protective bacteria and/or in the accumulation of colitogenic pathobionts, which leads to chronic inflammation [193] setting for CRC development.

Both dysbiosis and immune dysfunctions may allow increased bacterial translocation due to altered barrier function. Usually, gut microbes are recognized by the pattern recognition receptors (PRRs) which are specific for conserved microbial structures, such as nucleic acids or components of the bacterial cell wall [194]. According to the localization of PRRs, they can be divided in three major groups: extracellular, transmembrane and cytoplasmic PRRs (Fig. 17).

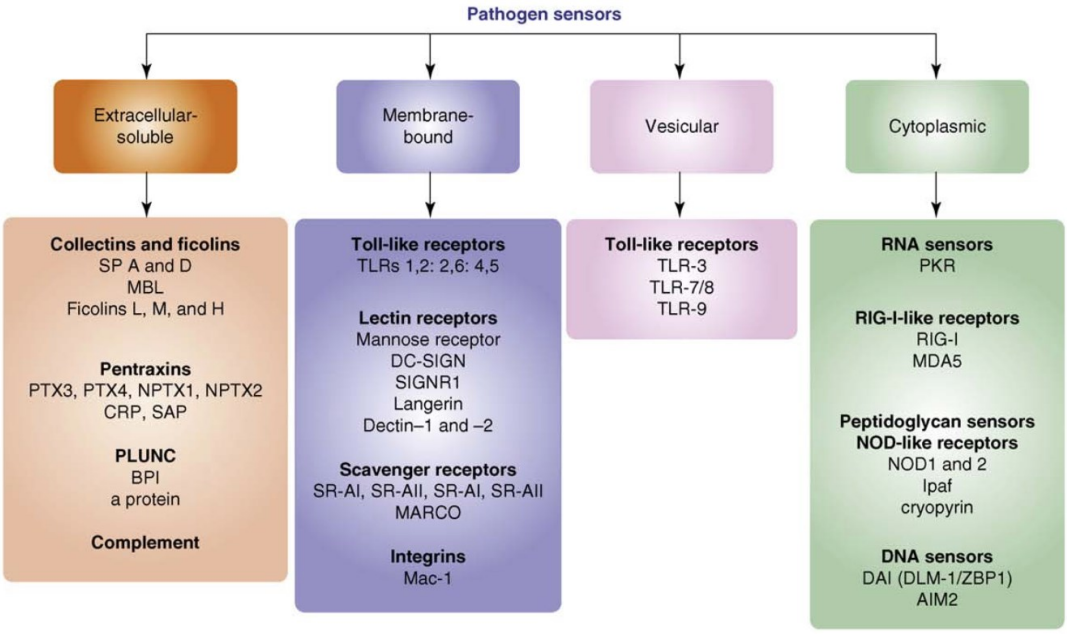


Fig. 17. *Pattern recognition receptors (PRRs) and their localization [195].*

In particular, the transmembrane PRRs include Toll-like receptors (TLRs) present in epithelial cells, macrophages, and myofibroblasts, leading to the activation of different pathways that influence, among other physiologic and pathologic conditions, cancer development [168].

2.5.1 Toll-like receptors

Toll-like receptors (TLRs) are a family of pattern recognition receptors. They have a key role in the first-line defence against pathogens by recognizing specific pathogen-

associated molecular patterns (PAMPs), conserved structures expressed by microbes and endogenous damage-associated molecular patterns (DAMPs). After ligand binding, TLR signalling leads to inflammation and antimicrobial responses, thus priming adaptive immune responses [196].

Human and mouse cells includes of 13 types of TLRs that can distinguish different kinds of bacterial and viral associated patterns. TLR1–9 are highly conserved in both species. The mouse TLR10 is non-functional due to retroviral insertion, TLR11–13 are undetected in the human genome [197]. Examples of TLR-specific microbial ligands are: lipopolysaccharide (LPS), endotoxin from Gram negative bacteria that stimulates TLR4; bacterial lipoproteins and lipoteichoic acid for TLR1, TLR2 and TLR6; bacterial flagellin which stimulates TLR5; unmethylated CpG motifs in DNA that activate TLR9; double-stranded RNA as activator of TLR3 and single-stranded RNA which stimulate TLR7 and TLR8 [198].

The signalling pathways orchestrated by TLRs are very intricate and have been studied over the past 15 years, as summarised in Fig. 18.

TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes. TLR4 localizes at both the plasma membrane and the endosomes [199].

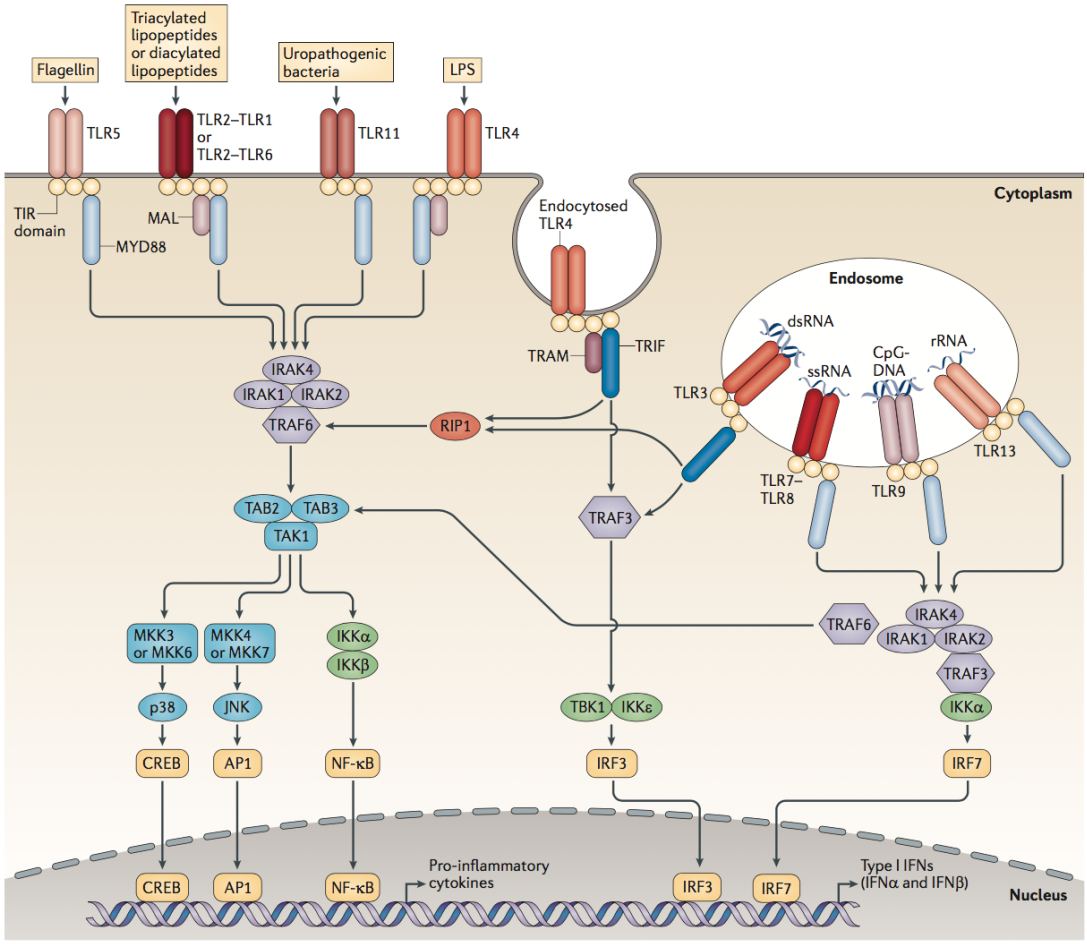


Fig. 18. *TLR signalling pathways* [199].
Myeloid differentiation primary-response protein 88 (MYD88); MYD88-adaptor-like protein (MAL); TIR domain-containing adaptor protein inducing IFNβ (TRIF); TRIF-related adaptor molecule (TRAM); IL-1R-associated kinases (IRAKs); adaptor molecules TNF receptor-associated factors (TRAFs); mitogen-activated protein kinases (MAPKs); JUN N-terminal kinase (JNK) and p38; nuclear factor-κB (NF-κB); interferon-regulatory factors (IRFs); cyclic AMP-responsive element-binding protein (CREB); activator protein 1 (AP1); interferon (IFN); double- stranded RNA (dsRNA); inhibitor of NF-κB kinase (IKK); lipopolysaccharide (LPS); MAP kinase kinase (MKK); receptor-interacting protein 1 (RIP1); ribosomal RNA (rRNA); single-stranded RNA (ssRNA); TAK1-binding protein (TAB); TGFβ-activated kinase (TAK); TANK-binding kinase 1 (TBK1).

In organs such as the lung, brain and colon, activation of TLR signalling at the steady state maintains tissue architecture, but in the presence of deregulated infection, inflammation and/or tissue injury as occurs during various stages of tumorigenesis, the unregulated TLR-regulated tissue repair response can orchestrate tumour growth and progression in a positive feedback of tolerant tissue injury and repair. Thus, TLRs may promote carcinogenesis through proinflammatory, anti-apoptotic, proliferative and profibrogenic signals generated in the tumor microenvironment or tumor cells themselves directly in TLR-expressing target cells, or mediated by TLR-induced cytokines

[198,200]. It has been shown that persistent TLR-specific activation of NFκB in CRC, and particularly in tumor-initiating cells, may sustain tumor growth and progression through perpetuation of inflammatory signaling and tissue repair mechanisms, with consequent self-renewal of pluripotent tumor cells. Thus, targeting of TLR signalling may be a possible mechanism to abolish this inflammation-mediated effect in tumor progression [201]. Recent studies of TLR-mediated modulation of immune response has led to novel immunotherapeutic strategies for the treatment of cancers [202]. Nevertheless, the true pathogenetic and therapeutic potential of TLRs signaling has not yet been realized [199].

2.5.1.1 TLR4 in CRC progression

As previously described, microbiota signaling has a pivotal role in intestinal colon cancer carcinogenesis. In particular, TLR4 was involved in several studies in the regulation of tumor growth, survival and progression connected to inflammation but its role is indeterminate and, sometimes controversial in non-inflammatory carcinogenesis [200].

TLR4 is overexpressed during inflammation-associated colorectal neoplasia in humans and mice. Similarly, mice lacking TLR4 are largely protected from colon carcinogenesis [203]. In fact, Tlr4 deletion strongly reduces inflammation and tumor burden in a colitis- associated neoplasia using the azoxymethane (AOM)-DSS model and transgenic mice overexpressing constitutively activated TLR4 in the intestine exhibit a higher sensitivity to colitis-associated neoplasia.

In contrast, recent studies showed that intestinal overexpression of constitutively activated TLR4 in the APC^{Min} model of colon reduces tumor load by increasing tumor cell apoptosis [200,204].

3 Aims

Mine project has been focused on the mechanisms of immunosurveillance in sporadic colorectal carcinogenesis, starting from the previously described expression of CD80 in human colon during the different stages of sporadic CRC and using:

- *in vivo* sporadic CRC carcinogenesis model;
- *in vitro* experiment.

Accordingly, we analyzed the role of the immune system in colon cancer prevention with particular interest in the activation of the CD80 costimulatory molecule on the surface of the epithelial cells.

Furthermore, in this contest in which microbiota is directly linked to intestinal colon cancer carcinogenesis, Toll-like receptors signaling are often under investigation for their capability to coordinate antitumor immunity. In particular we focused on:

- the role of TLR4 in non-inflammatory carcinogenesis;
- its activity in non-professional (i.e. IECs) and professional (i.e. DCs) antigen presenting cells to activate tumor T cells responses.

4 Materials and methods

4.1 Cell cultures and reagents

For the experiments *in vitro,* CT26.WT (ATCC CRL-2638TM) cell line was used. This undifferentiated colon carcinoma cell line was obtained from the colon of BALB/c mice treated with N-nitroso-N-metiluretano-(NNMU). Cells were grown till 80-90% confluence in DMEM medium (Gibco®) supplemented with 10% FBS v/v, sodium pyruvate 1% v/v and penicillin/streptomycin 1% v/v. Cells were kept in humified CO_2 (5%) incubator at 37 °C. The culture medium was renewed every 2-3 days and the cells were passaged with a trypsin/EDTA (Gibco®) solution and diluted in a ratio of 1:10 when they reached confluence.

Cell cultures were set up to perform a series of assays to quantify the expression of CD80 in response to Hydrogen Peroxide in presence or absence of pharmacological inhibitors.

N-Acetyl-L-cysteine (NAC) was purchased from Sigma – Aldrich (Milan, Italy). Hydrogen peroxide 10 volumes (3% solution) from Sella Srl (Schio, Italy). Pro-oxidant and antioxidant doses are listed in Table 5.

Table 5. *Pro-oxidants and antioxidant agents.*

Chemicals	Mechanism of action	Concentration
Hydrogen Peroxide	Chemical oxidation of cellular components	200 μM
N-acetyl cysteine	Source of SH group: stimulate glutathione synthesis and glutathione-s-transferase activity promoting detoxification and acts directly on free radicals	25 mM

Pharmacological inhibitors were used to inhibit pro-oxidants induced effect.

α-Amanitin was purchased from Cayman Chemical (Ann Arbor, MI), Caffeine (Anhydrous) from Bio Vision Inc. (San Francisco, CA), JSH-23 (inhibitor of NF-kB nuclear translocation), SB203580 (used to inhibit MAPK signalling), SP600125 (C-Jun N-terminal kinase inhibitor) and 5,15-DPP from Sigma – Aldrich (Milan, Italy), p38 MAP Kinase

Inhibitor X, BIRB 796 and another C-Jun N-terminal kinase inhibitor, AS601245 from Merck Millipore (Milan, Italy). Pharmacological inhibitors and doses are listed in Table 6.

Table 6. *Pharmacological inhibitors.*

Inhibitor	Target	Concentration
α-Amanitin	RNA polymerase II, III and IV	10 μg/ml
Z-vad-fmk	Caspase inhibitor	25 μM
JSH-23	NF-kB nuclear translocation	10 μM
Caffeine	ATM/ATR signalling inhibitor	5 mM
SB203580	p38 MAPK and MAPKAP kinase-2	5 μM
BIRB 796	p38 MAPK	5 μM
AS601245	JNK inhibitor	1 μM
SP600125	JNK Inhibitor	10 μM
5, 15-DPP	STAT3 inhibitor and selective STAT3-SH2 antagonist	5 μM

4.1.1 siRNA transfection

CT26 cell line was transfected with mouse-specific ATM/ATR, Trp53 and STAT3 siRNA (Origene Technologies, Rockville) and non-silencing siRNA (Silencer Negative Control siRNA) (Origene Technologies, Rockville). For transfection, 2 x 10^5 cells were seeded in each well of a 12-well plate (for RNA extraction) or 10^5 to each well of a 24-well plate (for flow cytometry analysis). The transfection was performed using the RNAimax Lipofectamine transfection agent (Invitrogen) and the siRNAs were used at a concentration of 10 nM. The plates were incubated in 5 % CO_2 at 37°C for 24 h; fresh medium was then added containing 200 μM H_2O_2 and the cells were incubated for additional 24h before the flow cytometry analysis and RNA extraction.

4.2 Isolation and culture of mice intestinal epithelial cells (IEC)

IEC cultures were set up, as previously described by Grossmann et al. [205], to perform a series of assays to quantify the expression of CD80 in response to stimulation with H_2O_2 for 24 hours.

Mice were sacrificed, the abdomen opened, and the colon removed. The lumen was extensively washed with ice – cold RPMI and flat opened. Following dissection of the mucosa into small strips and mucus removal by 1 mM DTT (Applichem) in HBSS 30 min at room temperature, mucosal strips were incubated in 1 mM EDTA for 10 min at 37°C. Then, mucosal strips were transferred into fresh culture medium (DMEM with 10% heat inactivated Fetal Bovine Serum (FBS), 2.5% penicillin-streptomycin-Fungizone and 1% gentamicin, all from Gibco). Tissues were vigorously shaken for 10 minutes (to facilitate the detachment of IECs in a full – length crypt formation). Then, the IEC crypts solution was transferred to a collagen I-coated (20 μg/cm^2, Sigma) 12-well plate for seeding of the cells.

4.3 Isolation and culture of bone marrow-derived dendritic cells (BMDC)

Mice were sacrificed and the posterior legs, above the hip joint, were removed. The muscles were detached, and the femurs were placed in 70% ethanol for 5 minutes. Both ends of the bone were removed using a sterile knife and the bones were flushed with ice-cold medium (RPMI with 2.5% penicillin-streptomycin-Fungizone and 1% gentamicin) until the bone marrow was completely collected. Bone marrow clusters were dissolved by pipetting and then washed by centrifugation (1-2 times at 1,500 rpm for 5 minutes).

The bone marrow cells were resuspended and diluted into 8-10 mL of complete medium (RPMI with 10% FBS, 2.5% penicillin-streptomycin-Fungizone, 1% gentamicin, 20 ng/mL GM-CSF and 20 μg/mL IL-4) and plated in Petri dishes at a density of around 2x10^6 viable cells per plate in a 37°C incubator with 5% CO_2. GM-CSF and IL-4 were purchased from Immuno Tools, Oldenburg, Germany.

An additional 8-10 mL of fresh medium was added at day 3.

At day 6 and at day 8 half of the media was removed, briefly centrifuged, resuspended in 8-10 mL of fresh medium and added to the original culture.

The immature DCs (around the 70%) were used at day 9 and treated with Lipopolysaccharides (LPS) from Salmonella for 24 hours (Sigma – Aldrich, Milan, Italy).

4.4 Mouse colon cancer model

Animal experiments were performed according to Italian Law 116/92 and European directive 2010/63/UE. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee ("Comitato Etico Scientifico per la Sperimentazione Animale") of the University of Padova, Padova, Italy and by the Ministry of Health. Mice were maintained under standard laboratory conditions with 12:12-h light-dark cycles and free access to regular rodent chow food and water at all stages of the experimental protocol.

For the study C57Bl6/J (Wild Type), B6.129S4-Cd80 tm1Shr /J ($CD80^{KO}$) and B6.B10ScN-Tlr4lps-del/JthJ ($Tlr4^{KO}$) male mice were used. Cohoused 12-wk-old mice were injected with azoxymethane (AOM, Sigma – Aldrich, Milan, Italy) i.p. at a dose of 10 mg/kg body weight (mean weight 27 ± 1gr) once a week for six consecutive weeks. All mice were housed in the same animal room and sacrificed at different time points (4^{th} month, 6^{th} month and 8^{th} month) after the first AOM injection.

Some WT mice received two doses i.p. of a monoclonal anti-CD80 antibody (clone 16-10A1, ATCC hybridoma no. HB-301) (200 ug/mice) at the 3^{rd} and the 4^{th} following AOM administration.

Following sacrifice, the abdomen was opened, and colon was carefully removed and flushed with ice-cold PBS to remove faeces. Each colon was flat opened along the mesentery and visually inspected for the presence of macroscopic lesions (i.e. adenomas) and pictures were taken with a digital camera. A small segment of the most distal colon above the anus (proximal to the cecum) was cut and snap frozen for subsequent total RNA extraction (see below). Then, the colon was cut longitudinally to obtain two tissue strips. One strip was processed for cytofluorimetric analysis (see below), whereas the remaining tissue was fixed in 10% neutral-buffered formalin. After 24 hours tissues were paraffin embedded as "Swiss rolls".

4.4.1 Monoclonal antibodies purification

Monoclonal antibodies were produced by hybridomas in suspension culture in Iscove's Modified Dulbecco's Medium containing 10% heat inactivated fetal bovine

serum (FBS). Anti-CD80 monoclonal antibody was produced by clone 16-10A1 (ATCC hybridoma no. HB-301).

The culture supernatant was collected by centrifugation, diluted 1:1 with binding buffer (phosphate-based contain EDTA, pH 8.0) and monoclonal antibodies purified using a Protein G PLUS-Agarose column (Santa Cruz Biotechnology, Italy). Protein concentration was determined by the Bradford method with a commercially available kit (Protein Assay Kit; Bio-Rad Laboratories, Hercules, CA).

4.4.2 BM transplantation in mice

Six-week old C57Bl/6J WT or $CD80^{KO}$ recipient mice were irradiated with 900 Rad. BM cells were isolated from 6-8 week old C57Bl/6J WT or $CD80^{KO}$ donor mice by flushing the bone shafts of the femurs and tibias with RPMI and 10^7 BM cells i.v. injected into the recipient mice after irradiation. WT mice were transplanted with CD80-/- BM ($CD80^{KO}$ BM → WT) and $CD80^{KO}$ mice were transplanted with WT BM (WT BM → $CD80^{KO}$). As controls, WT mice were transplanted with WT BM and $CD80^{KO}$ mice transplanted with $CD80^{KO}$ BM. Six weeks after transplantation BM chimeric mice were injected with 10 mg/kg of AOM i.p. once a week for 6 weeks.

4.5 Histopathology

Three µm sections were cut from formalin-fixed and paraffin - embedded mice colon specimens. Sections were air – dried on regular glass slided and stained with haematoxylin-eosin. Histological inflammation was quantified and classified by a pathologist unaware of the arm of the experiment using Floren's score and the Vienna classification of gastrointestinal epithelial neoplasia.

The extent of dysplasia was quantified as the percentage of involved bowel length.

4.6 Immunohistochemistry

Immunohistochemical analyses were performed using standard procedures.

Sections (4 µm thick) from paraffin embedded tissue samples were deparaffinised using Xylene (Carlo Erba Reagents, Milan, Italy) and rehydrated with scalar

concentration of ethanol (100%, 95%, 70%, 5 min for 2 times). Endogenous peroxidase activity was blocked (10% H_2O_2 for 5 min) before antigen retrieval (citrate buffer, pH 9, 30 min at 95°C). Tissues were then placed in universal blocking solution (Lab Vision Corporation, CA, USA) and then incubated with the proper primary antibody for 1 hour at 22°C. Unbound antibody was removed by extensive tissue washing. The immunocomplexes were detected using the Real Dako Envision System detection system (Dako, Glostrup, Denmark).

The Immunohistochemical staining was performed using the polyclonal antibody to CD80 indicated in Table 7. The reaction was highlighted through the use of the chromogenic substrate 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark). The sections were counterstained with Mayer's haematoxylin, subjected to dehydration in increasing solutions of alcohols and xylene, and finally mounted in Dako Mounting Medium. The preparations were observed under an optical microscope and immunohistochemical evaluation was carried out in at least 10 fields (20X magnification).

Table 7. *IHC antibody anti-mouse CD80.*

Antibody	Company	Dilution
Anti-CD80 polyclonal antibody	Bioss Antibodies Inc., Massachusetts, U.S.A.	1:200

4.7 Flow cytometry

Flow cytometry analysis was performed on cultured cells and primary cells isolated from experimental mice.

In vitro cultured cells treated with appropriate stimuli and/or inhibitors were tripsinized, washed with 1X PBS and incubated with anti CD80 mouse FITC-conjugated (Immunotools, Oldenburg, Germany), anti MHC II mouse PE-conjugated or anti CD11c PE-Cy7 antibody (from eBioscience Inc., San Diego, CA) for 30 mins at 4°C. After this step, cells were washed with 1X PBS and transferred to flow cytometry tubes.

The thinner colonic strips (see paragraph 4.4) obtained from the different experimental animals were extensively washed in ice-cold PBS to remove any faecal

residue and then minced into 3 to 4 mm pieces with a sterile scalpel. Tissues were incubated in HBSS supplemented with 1 mM DTT and 0.5 mM EDTA with shaking at 37 °C for 20 min, to remove mucus and debris. After washing, tissue pieces were treated with 1 U/ml Dispase (Stemcell Technologies) in HBSS at 37 °C for 30 min with gentle stirring. Tissues were then collected and filtered through a sterile stainless-steel mesh (pore size 80 µm, Sigma-Aldrich, Milan, Italy) in order to obtain a single-cell suspension. Cells were collected by centrifugation, resuspended in PBS, counted, allocated to be stained with appropriate combinations of FITC- and PE- conjugated antibodies for 30 mins at 4°C.

Single-cell suspensions were subjected to flow cytometry to determine the proportion of epithelial cells (Cytokeratin pan) expressing CD80, MHC I and MHC II), the proportion of activated CD8+ T cells (positive for CD28 and CD38) and the proportion of activated CD4+ T cells (positive for CD25). The antibodies used are listed in Table 8.

Table 8. *Antibodies used in flow cytometry.*

Antibody	Company
Anti-mouse pan Cytokeratin PE	Abcam Ltd., UK Cambridge
Anti-mouse CD80 FITC	Immunotools, Oldenburg, Germany
Anti-mouse MHC I FITC	eBioscience Inc., San Diego, CA
Anti-mouse MHC II PE	eBioscience Inc., San Diego, CA
Anti-mouse MHC II FITC	eBioscience Inc., San Diego, CA
Anti-mouse CD8a PE-Cy7	eBioscience Inc., San Diego, CA
Anti-mouse CD28 FITC	Abcam Ltd., UK Cambridge
Anti-mouse CD38 FITC	eBioscience Inc., San Diego, CA
Anti-mouse CD4 PE-Cy7	eBioscience Inc., San Diego, CA
Anti-mouse CD25 PE	eBioscience Inc., San Diego, CA
Anti-mouse Cd11c PE-Cy7	eBioscience Inc., San Diego, CA

Flow cytometric analysis was performed using a FACSCalibur based on CellQuest software (Becton Dickinson).

4.7.1 Detection of apoptosis using the annexin V FITC assay

The apoptosis detection was performed following the manufacturer's instruction manual (Annexin V-FITC Apoptosis detection Kit, eBioscence).

Cells were washed in PBS by gentle shaking and resuspend in 200 µl Binding Buffer (1X). Then, 5 µl Annexin V-FITC were added to 195 µl cell suspension. Cells were mixed and incubated for 10 min at room temperature. After a 200 µl Binding Buffer (1x) washing, cells were resuspended in 190 µl Binding buffer (1X) and 10 µl Propidium Iodide (20 µg/ml) were added.

Samples were then subjected to flow cytometry analysis.

4.8 Purification of RNA and reverse transcription

Isolation of RNA from CT26 and mice specimens was performed following the manufacturer's instruction manual (SV Total RNA Isolation System, Promega, Wisconsin, USA).

Sample was prepared for lysis into 175µl RNA Lysis Buffer (+ β-mercaptoethanol) in an autoclaved tube and mixed thoroughly by inversion. Then, 350 µl of RNA Dilution Buffer was added and mixed by inverting 3-4 times before being heated at 70°C for 3 min. Colonic tissue samples (≤30 mg) were lysed and homogenized in RNA Lysis Buffer (+ β-mercaptoethanol) using a Retsch MM300 mixer.

Next, sample was centrifuged at maximum speed for 10 minutes and the cleared lysate transferred to a fresh tube added with 200 µl 95% ethanol. The mixture was transferred to Spin Basket Assembly and centrifuged at 1300 rpm for one minute, consequently the eluate was discarded. 600 µl of RNA Wash Buffer was added and centrifuged at 1300 rpm for another one minute. After this point was performed DNase incubation with the addition of 50 µl DNase mix to the membrane of the spin and incubated at room temperature for 15 min. 200 µl of DNase Stop Solution were added to membrane after the incubation and spin was centrifuged at 1300 for one minute. After that, washes with 600 µl and 250 µl of RNA Wash Buffer by centrifuge at 1300 rpm

for another one minute were performed. At the end, 50 µl of Nuclease-Free water were used to eluate the RNA.

Total RNA was stored at -80°C.

Reverse transcription is the process by which a reverse transcriptase enzyme converts RNA into complementary DNA (cDNA). For reverse transcription, reagents provided by Biorad (California, USA), *iScript*™ cDNA Synthesis Kit, were used. An amount of about 500 ng of total RNA was used for the reaction protocol that consist in an initial priming phase (5 min at 25 °C) followed by the reverse transcription phase (20 min at 46 °C) and the RT inactivation (1 minute at 95 °C).

4.9 Quantification of mRNA levels by RT-PCR

Levels of specific mRNA were measured by Real Time RT-PCR. The cDNA template was amplified in the quantitative step, during which the fluorescence emitted by intercalating dyes increases as the DNA amplification process progress.

Real time PCR reaction have been carried in a 20µl of volume with 10 µl SYBER Green PCR Master Mix, 300 nM of primers mix and 2 µl of cDNA template. Gene specific primers were designed using conventional parameters and specificity of the primers were determined by comparison to the Gene Bank database using the Based Local Alignment Search Tool (BLAST). In particular, primers paired two different exons.

To study the expression of the genes of interest, we used primers described in Table 9, and murine 18S was used for normalization as an internal control.

ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used. Data are presented as a mean fold change over the control.

Table 9. *Primers used in Real time PCR.*

Gene	Sequence 5'→3'	Tann, °C	Amplicon, bp
18S	FW CTTAGAGGGACAAGTGGCG RV ACGCTGAGCCCAGTCAGTGTA	60	108
Atm	FW GGAACCAGTTACCATGAATCGTT RV TCTTCAACTTCTTTCACCCTGA	60	110

Atr	FW AGCAAGGTGATCTCATCCGA RV CGACCACCTTTTTCCCATTCG	60	148
Batf3	FW CAGACCCAGAAGGCTGACAAG RV CTGCGCAGCACAGAGTTCTC	66	71
CD80	FW CCCCAGAAGACCCTCCTGATAG RV CCGAAGGTAAGGCTGTTGTTTG	62	172
Cdkn1a	FW CTGACTCCAGCCCCAAACAT RV CAGGGAGGGAGCCACAATAC	60	100
Nrf2	FW AGATGACCATGAGTCGCTTGC RV CCTGATGAGGGGCAGTGAAG	60	74
Prdx2	FW GACCTACCTGTGGGACGCTC RV CCACATTGGGCTTGATGGTGT	60	130
Prdx6	FW CTCCAGCTGACAGGCACAAA RV TCGGAGAGGGTGGGAACTAC	60	86
STAT3	FWACTTCAGACCCGCCAACAAA RV CACCACGAAGGCACTCTTCA	60	148
Trp53	FW CGACTACAGTTAGGGGGCAC RV ATGGCAGTCATCCAGTCTTCG	60	93
Xcr1	FW CCTACGTGAAACTCTAGCACTGG RV AAGGCTGTAGAGGACTCCATCTG	66	136

4.10 Isolation of DNA from stool for 16S sequencing

Isolation of genomic DNA from mice stool was performed using a commercially available extraction kit following the manufacturer's instruction manual (QIAmp DNA Stool mini kit, Qiagen, Venlo, Netherlands).

Mice stool samples were obtained placing animals separately in clean cages and quickly collecting 4-5 faecal pellets (180-220 mg). Stools were lysed and homogenized in ASL Buffer (+ β-mercaptoethanol) using a vortex. Then, the suspension was heated for 5 min at 70°C, vortexed for 15s and centrifuged at maximum speed for 1 minute. 1,2 ml of the surnatant was transferred in a new microcentrifuge tube with one InhibitEX Tablet and continuously vortexed until the tablet was completely suspended. Then, the suspension was incubated at room temperature for one minute and centrifuged at full speed for 3 min. All the supernatant was placed into a new 1.5 ml microcentrifuge tube, centrifuged at full speed for other 3 min and 200 μl of this supernatant were placed into

a 1.5 ml microcentrifuge tube added with 15 μl proteinase K. Next, 200 μl of Buffer AL were added and vortexed before another incubation at 70°C for 10 min.

Later, 200 μl of ethanol (96-100%) were supplemented to the lysate and mixed by vortexing. After this point, the lysate was transferred to the QIAamp spin column and centrifuged at maximum speed for 1 min (the filtrate was discarded). 500 μl of Buffer AW1 were added and centrifuged for 1 min, then 500 μl of Buffer AW2 were added and centrifuged for 3 min. At the end, 50 μl of Nuclease-Free water were used to eluate the DNA.

DNA was stored at -20°C.

4.10.1 16s sequencing and data analysis

The analysis of 16S was entrusted the management to IGA Technology Services S.R.L., Udine, Italy.

They performed an initial PCR amplification using locus specific PCR primers and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). This method is used to amplify the variable V3 and V4 regions of the 16S rRNA gene to characterize bacterial community composition. The amplification is due to the following target sequences **16S-341F** 5'-CCTACGGGNGGCWGCAG-3' and **16S-805R** 5'- GACTACHVGGGTATCTAATCC-3' for the 16S locus.

Libraries are sequenced in a MiSeq run in paired end with 300-bp read length or HiSeq2500 with 250bp read length depending on the experiment type.

4.11 Immunofluorescence assays

To perform Immunofluorescence analysis, CT26 were seeded on coverslip slides placed in 12-wells plates. Following appropriate stimulation and/or treatments, cells were washed with PBS 1X (5 min) and fixed with PFA 4% for 10 min. Finally, cells were washed with PBS 1X (5 min). To detect CD80, cells were incubated with antibodies against CD80 (Table 10) for 1 h at 37°C without permeabilization after 40 min in blocking

solution (Bovine Serum Albumin, BSA, 1% in PBS1X). Then, cells were washed with PBS 1X and slides were mounted using glycerol.

To detect histone H2A.X and NF-κB p65, after the previously described fixation step with the PFA, cells were incubated in PBS-Triton 100X 1%, for 10 min for membrane permeabilization. Then, cells were washed with PBS 1X (5 min) and covered by the blocking solution for 40 min. After that, cells were incubated with appropriate primary antibody over night at 4°C. Then, slides were washed (3 times for 5 minutes) in PBS 1X and incubated with Alexa Fluor 488 anti–rabbit (dilution 1:200 from Jackson Immuno Research Laboratories, West Grove, PA, USA) for 1 hour at room temperature. The slides were washed, counterstained with DRAQ5^{TM} (Thermo Fisher) fluorescent probe solution to identify nuclei and mounted using glycerol.

Table 10. *Antibodies used in immunofluorescence assays.*

Antibody	Company	Dilution
Anti-mouse CD80 FITC	eBioscience Inc., San Diego, CA	1:100
Anti Histone H2A.X	Genetex, Inc., North America	1:500
Anti-NF-kB p65	Santa Cruz Biotechnology, Texas, USA	1:1000

Slides were analysed with a confocal laser scanning microscope (Nikon A1R-A1 or Leica TCS-NT/SP2). Image analysis was performed using the Nikon A1R-A1 and Leica TCS-NT/SP2 software.

4.11.1 Qualitative detection of mitochondrial superoxide and ROS production in live cells

CT26 cells seeded on slide coverslips were incubated with complete medium with or without hydrogen peroxide for 30 min or an overnight treatment. To test the presence of ROS, living cells were incubated with 5μM MitoSOX [3,8-phenanthridinediamine, 5-(6'-triphenylphosphoniumhexyl)-5,6 dihydro-6- phenyl] or 5μM CM-H_2DCFDA (all purchased from Molecular Probes, Invitrogen, Carlsbad, CA) for 30 – 60 mins in CO_2 incubator at 37 °C. Then, cells were washed with PBS 1X and fixed with PFA 4%. Slides were mounted using glycerol and analyzed with a confocal laser scanning microscope.

4.12 Western blotting

4.12.1 Sample preparation, running the gel and protein transfer

CT26 cells were homogenized in RIPA buffer, radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS -sodium dodecyl sulphate-, protease inhibitors and sodium orthovanadate 1mM). Particulate material was removed by centrifugation at 1300 rpm at 4°C. Protein concentration was determined in each sample using PierceTM BCA protein assay kit (ThermoFisher SCIENTIFIC, Massachusetts, United States). Twenty µg protein were loaded into the SDS-polyacrylamide gel (10%), along with molecular weight markers. Then transferred onto a nitrocellulose membrane (0.45 µm pore size in roll form, Millipore) and care was taken to remove all air bubbles.

4.12.2 Antibody staining

The electrophoretic blots were blocked in 5% bovine serum albumin (BSA) or 5% milk in TBST (120 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) for 1 hr at room temperature to saturate additional protein binding sites. Then membranes were incubated overnight a 4°C with primary antibody following the manufactured data sheets. After membranes washing with TBST 0.1% Tween 20 (about 3 times for 5 minutes), they were incubated with the proper horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using Clarity^{TM} western ECL blotting substrates, Biorad and images were captured using the Calestream® Kodak® Biomax® light films (Sigma – Aldrich, Milan, Italy) and the Blue X Ray films (Aurogene, Rome, Italy). To ensure equal loading and accuracy of changes in protein abundance, protein levels were normalized to β - actin as housekeeping.

Primary antibodies used in Western Blotting are listed in Table 11.

Table 11. *Primary antibodies used in Western blotting.*

Antibody	Company	Dilution
Anti pSTAT3 Rabbit	Cell Signaling Technology, Massachusetts, USA	1:2000
Anti β - actin Mouse	Sigma Aldrich, Milan, Italy	1:5000

4.13 HPLC and GC-MS

HPLC and gas chromatography analysis were performed by Prof. Stefano Dall'Acqua at the Department of Pharmacy, University of Padua, and by Prof. Bogumila Szponar, Institute of Immunology and Experimental Therapy Polish, respectively.

In particular, for HPLC-MS/MS analysis, 10-50 mg of mice colonic mucosa were weighted, extracted with a mixture of 100 µl trichloroacetic acid containing 10% EDTA 1mM, centrifuged at 13000 rpm and clear supernatant were collected. Samples (up to 20 µl) were finally injected. A Phenomenex Kinetek F5 (50 x 3 mm) 2,6 micron column was used. The mobile phases were 1% formic acid in water and methanol. Separation was performed under gradient conditions from 95% of water to 95% methanol.

Stock solutions of GSH and GSSG were prepared at 1 mM in 10% TCA solution and stored at -80°C. Calibrators containing both GSH and GSSG at different concentrations were prepared fresh on the day of the experiment and run to validate the method.

4.14 Statistics

Data are shown as mean +/- SEM. Mann–Whitney's U-test and t-test student were performed using GraphPad Prism Software 6.0 (GraphPad Software Inc., La Jolla, USA). Differences were considered significant at $p<0,05$: *$p<0,05$; **$p<0,01$; ***$p<0,001$.

5 Results

5.1 CD80 controls the progression of colonic preneoplastic lesions

Given that CD80 expression is increased in the epithelial compartment of human colonic preneoplastic lesions, we chose to investigate its functional relevance in mice. We used the mutagenic agent azoxymethane, known to induce colonic tumors in certain strain of mice. Among mice strains, C57BL/6 mice are resistant to AOM-induced tumors, thus representing an ideal model to study preneoplastic lesions development. Indeed, in wild-type C57BL/6 (WT) mice AOM treatment resulted in low grade dysplasia after 4 and 6 months from the first injection.

We next characterized CD80 expression in the AOM mouse model of colorectal cancer. As shown in Fig. 19A immunohistochemical staining showed that CD80 is expressed not only by lamina propria mononuclear cells, but also by dysplastic intestinal epithelial cells of AOM-treated mice. Moreover, flow cytometric analysis on CEC (Fig. 19B) showed a significant increase in panCk+CD80+ cells in mice treated with AOM vs untreated mice suggesting a substantial induction of CD80 expression in the early stages of colonic carcinogenesis.

To determine the functional relevance of CD80 in the early stages of colonic carcinogenesis we used CD80 knockout ($CD80^{KO}$) mice and the administration of neutralizing monoclonal anti-CD80 antibody. In both models the lack of functional CD80 caused a significant increase in total dysplasia extension in AOM treated mice (Fig. 19C).

To further characterize the relevance of CD80 expressed on hematopoietic and non-hematopoietic cells, we generated $CD80^{KO}$/WT bone marrow chimeras. In these experiments $CD80^{KO}$ mice receiving bone marrow from WT mice are not able to express CD80 in epithelial cells but present CD80 on haematopoietic-derived cells, such as DCs and macrophages in the intestinal lamina propria. Data obtained from this experiment revealed a significant decrease in total dysplasia extension in mice lacking epithelial CD80 compared to controls (Fig. 19D).

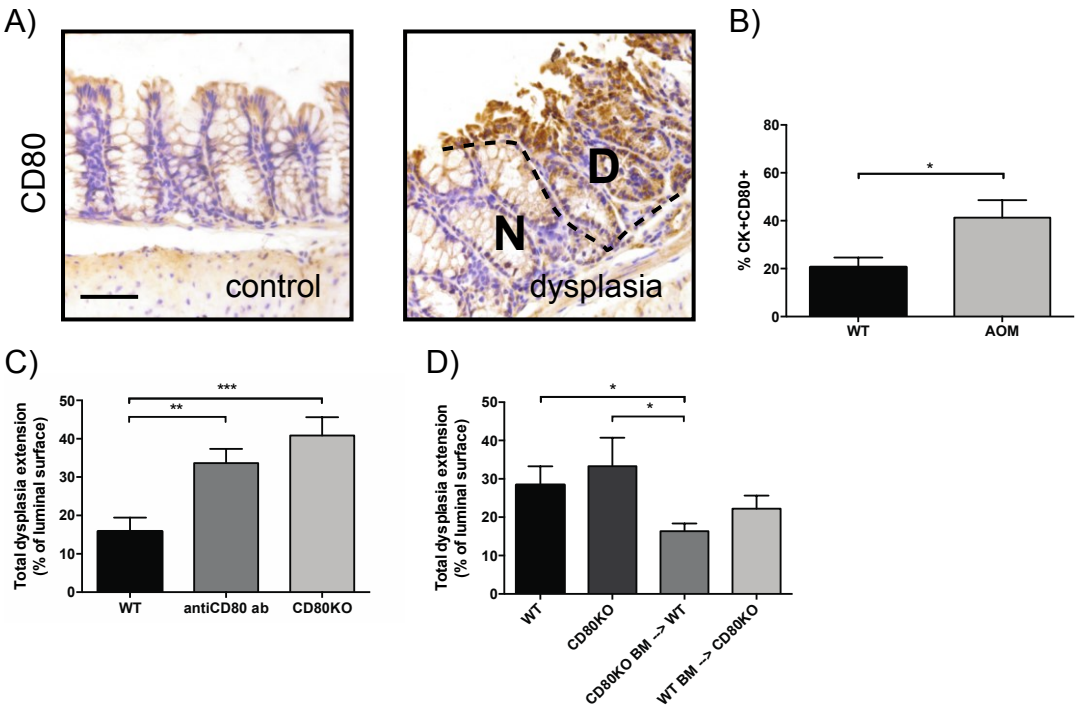


Fig. 19. *CD80 controls the progression of colonic preneoplastic lesions in AOM carcinogenesis model.*
A) Representative immunohistochemical staining of CD80 on the colon from control and AOM treated mice. CD80 is expressed not only by lamina propria mononuclear cells, but also by dysplastic intestinal epithelial cells of treated mice. B) Flow cytometry analysis of CD80+ epithelial cells (pan-ck+). C) CD80 deficient mice are more susceptible to AOM preneoplastic lesions. Total dysplasia extension of $CD80^{ko}$ mice and WT treated with antiCD80 antibody represented. D) Epithelial CD80 expression contributes to inhibition of dysplasia development. Total dysplasia extensions of $CD80^{ko}$/WT bone marrow chimeras are represented.
The Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for comparisons (n=7-21 mice per group).

5.2 Oxidative stress increases CD80 expression in colonic epithelial cells

Our results suggest that CD80 expression is induced in preneoplastic AOM-induced epithelial degeneration lesions, representing an early protective mechanism against CRC onset. Actually, it is known that AOM causes pathological changes in the colonic mucosa by increasing oxidative stress and consequently inducing genotoxicity, in view of that our hypothesis is that CD80 expression is upregulated during the early stages of colonic carcinogenesis by ROS [206–208].

We firstly assessed the oxidative stress balance in the colonic mucosa. AOM-treated colonic mucosa was characterized by an oxidative microenvironment, as shown by a significant downregulation of the mRNA transcripts for antioxidant genes Nrf2,

Prdx2 and Prdx6 (Fig. 20A) and a consistent reduction in the ratio of reduced GSH to oxidized GSH (GSSG) (Fig. 20B), as compared to untreated colonic mucosa.

To investigate the role of oxidative stress on CD80 expression, we first attempted to generate an appropriate cellular model *in vitro.* Therefore, we assessed the ability of a pro-oxidant agent (H_2O_2) to generate oxidative stress in cells. To this end we stained untreated and pro-oxidant treated cells with two fluorogenic dyes, which are selective indicators of mitochondrial superoxide and ROS, respectively. Confocal microscopic imaging demonstrated a significant increase in mitochondrial superoxide and ROS production in cells treated for 30 mins or overnight with pro-oxidant (Fig. 20C).

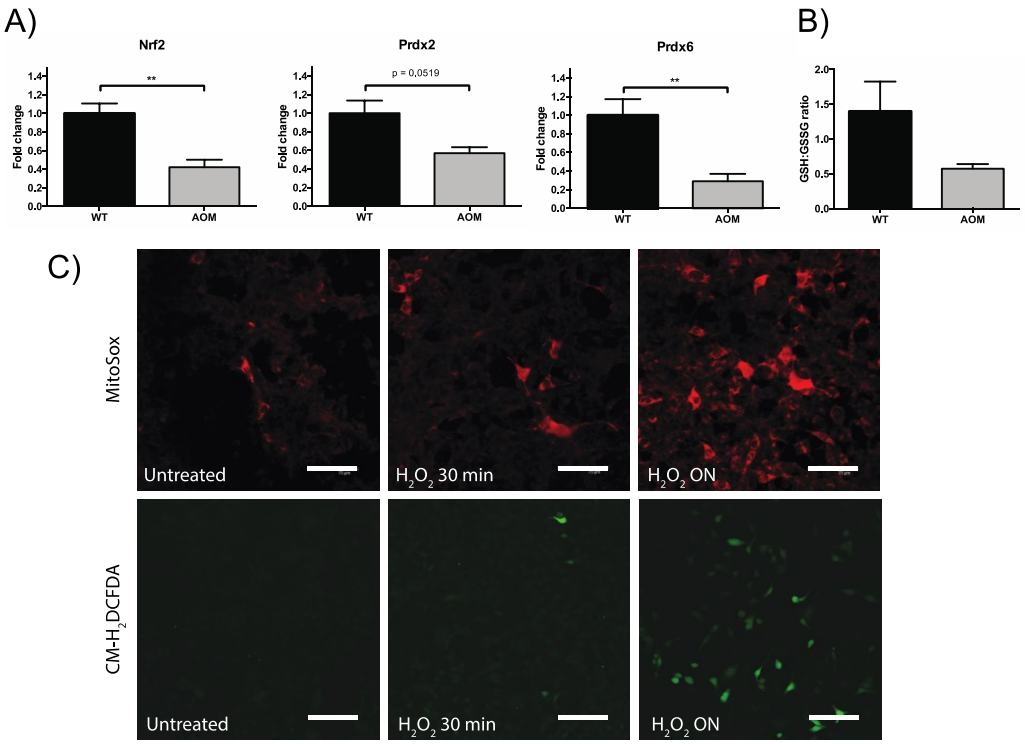


Fig. 20. *Oxidative microenvironment in the AOM model and in CT26 cell line.*
A) *AOM model shows dysregulation of oxidative stress genes as shown by relative Nrf2, Prdx2 and Prdx6 mRNA expression after AOM treatment measured by RT-PCR expressed as fold change using 18s as endogenous control. Data represent mean ± SEM (n=5-6). The Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for comparisons). B) Increased oxidative stress levels measured as reduced/oxidized GSH by HPLC. Data represent mean ±SEM (n= 7-4). C) H_2O_2 increased mitochondrial superoxide and ROS generation in cells. Representative confocal images showing increase in mitochondrial superoxide production (red fluorescence) and ROS generation (green fluorescence) following 30 mins or overnight pro-oxidant treatment. Magnification 40X.*

In order to verify the contribution of ROS to the induction of CD80 expression in CEC, we exposed CT26 cells to H_2O_2. Treatment with H_2O_2 significantly increased CD80 expression as measured by flow cytometry and immunofluorescence assay (Fig. 21A and 21B). We also verified that exposing CT26 to an oxidative stress induced CD80 up-regulation inducing gene transcription. Indeed, H_2O_2 treatment significantly increased CD80 mRNA level as detected by RT-PCR. Moreover, CT26 treatment with α-amanitin (that is known for its ability to inhibit RNA polymerase II) abolished H_2O_2 treatment – induced CD80 up – regulation (Fig.21C).

To further demonstrate the role of the oxidative stress induced by pro-oxidants in the increase of CD80 expression, we pre-treated cells with N-acetylcysteine (NAC), an antioxidant agent, before exposing cells to H_2O_2. Flow cytometry analysis of CT26 cells indicated that N-acetylcysteine successfully decreased H_2O_2-induced CD80 expression (Fig. 21D). Moreover, to confirm the key role of free radicals on colonic epithelial cell expression of CD80 *in vivo* during colonic carcinogenesis, we directly tested the effect of an oxidative stress on primary colonic epithelial cells. As reported in Figure 23E, primary colonic epithelial cells exposed to an oxidative stress (i.e. H_2O_2) significantly increased CD80 expression corroborating pro-oxidant agents as a key regulator of CD80 (Fig. 21E).

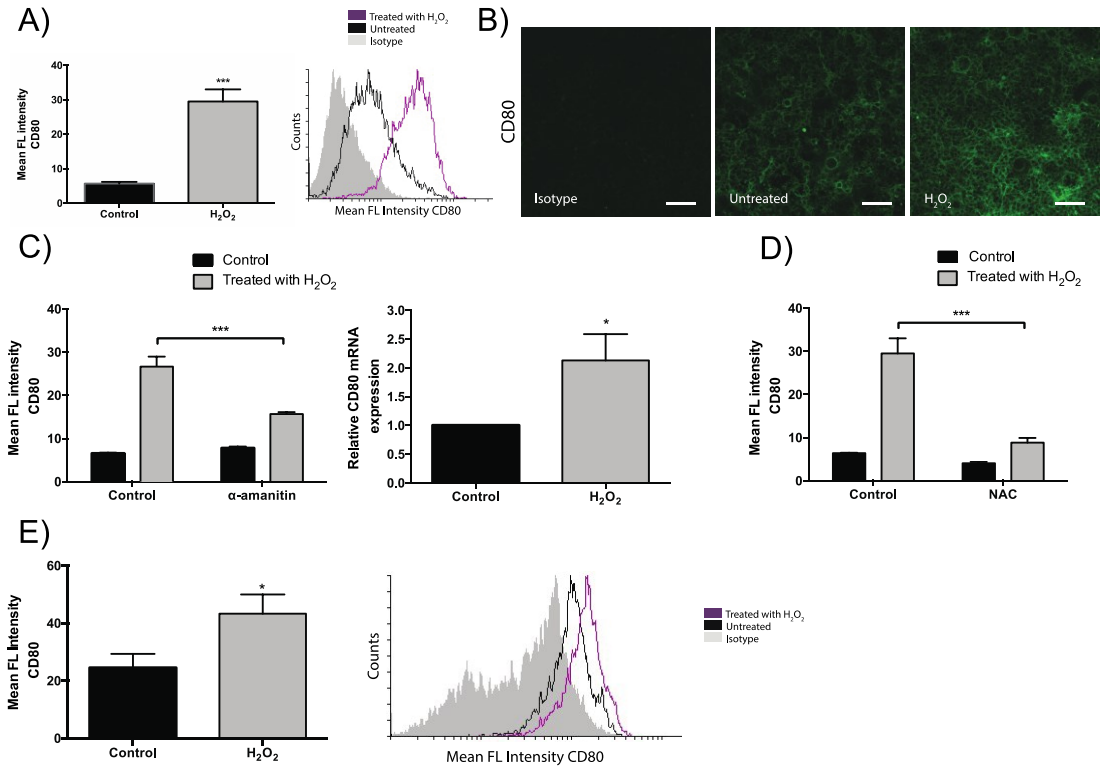


Fig. 21. *H_2O_2 treatment increased CD80 expression.*
A) Quantitative data showing the increase of CD80 expression following H_2O_2 24 hours' treatment measured by flow cytometry. Data are representative of three independent experiments (n=6). Filled histogram correspond to an isotype control, continuous black line corresponds to untreated CT26 cells and continuous violet line corresponds to CT26 treated with H_2O_2. B) Representative confocal images showing increase in CD80 expression (green fluorescence) following overnight H_2O_2 treatment. Magnification 20X. C) Quantitative data expressing inhibition of H_2O_2 induced CD80 expression after α-amanitin treatment (flow cytometry assay). Data are representative of three independent experiments (n=6). Relative CD80 mRNA expression after H_2O_2 treatment expressed measured by RT-PCR as fold change using 18s as endogenous control. Data represent mean $\pm$ SEM (n= 4) and are representative of four independent experiment. D) Antioxidant pre-treatment with N-acetyl-cysteine (NAC) suppressed the positive effect of H_2O_2. Quantitative data expressing changes of CD80 expression after H_2O_2 and NAC treatments measured by flow cytometry. Data are representative of three independent experiments (n=6). E) Quantitative data showing the increase of CD80 expression after H_2O_2 treatment in primary colonic epithelial cells. CD80 expression was measured by flow cytometry in primary colonic epithelial cells positive for cytokeratin after 24 hours from treatment. Data are representative of four independent experiments (n=8). Filled histogram correspond to an isotype control, continuous black line corresponds to untreated primary IECs and continuous violet line corresponds to IECs treated with H_2O_2.
The ordinary one-way ANOVA followed by unpaired T test with equal SD were used for comparisons.

5.2.1 CD80 induction by oxidative stress is not a consequence of apoptosis or NF-kB signalling

Previous studies showed that following exposure to H_2O_2, CEC are primed for cell death. Thus, it is possible that the induction of CD80 could be a result of oxidative-stress induced apoptosis we quantified the amount of H_2O_2 treatment – induced apoptosis in our *in vitro* model. In our experiments, 24h treatment with 200 µM H_2O_2 did not cause a significant increase in apoptotic cells, as determined by Annexin V+ CT26 cells (Fig. 22A). Moreover, we blocked caspase activation using a pan-caspase inhibitor, Z-VAD-fmk. This treatment did not prevent H_2O_2-mediated CD80 induction (Fig. 22B), thus ruling out the possibility that CD80 expression is a consequence of apoptosis.

Nuclear factor-kappaB (NFκB) signalling is another of the key regulatory pathways classically activated by oxidative stress that could be involved in CD80 induction. We pharmacologically blocked NF-κB nuclear translocation using the inhibitor JSH-23 in H_2O_2-treated CT26 cells. As expected, JSH-23 inhibited LPS-induced nuclear translocation of the p65 subunit of NF-κB as analysed by immunofluorescence assay (Fig. 22C).

However, H_2O_2-induced CD80 up-regulation in CEC was not affected, suggesting that CD80 induction is not NF-κB signalling dependent (Fig. 22D).

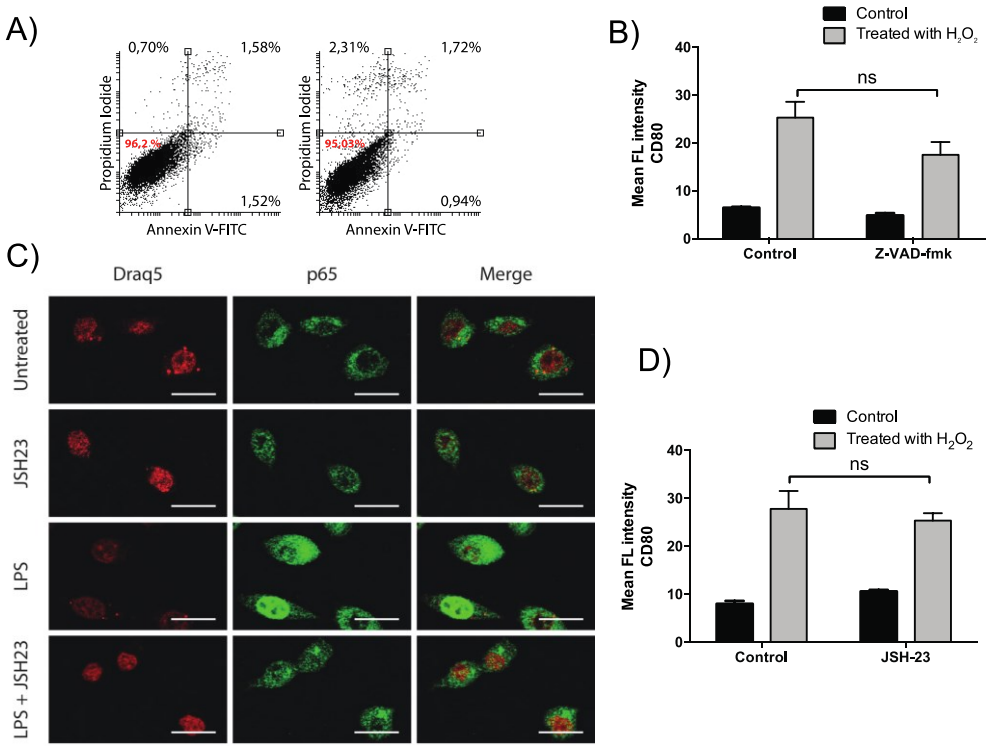


Fig. 22. *CD80 induction by oxidative stress is not a consequence of apoptosis or NF-κB signalling in CT26 cell line.*
A) H_2O_2 does not induce apoptosis in CT26 cell line after 24 hours treatment as analysed by flow cytometry. Legend: left bottom viable cells, right bottom early apoptotic cells, up right late apoptotic cells, up left necrotic cells. B) Z-vad-fmk inhibitor did not prevent H_2O_2 CD80 up regulation, quantitative data measured with flow cytometry. Data are representative of two independent experiments (n=4). C) JSH-23 inhibits p65 nuclear translocation in CT26 cell line, representative confocal images showing p65 subunit nuclear translocation (green fluorescence) following 30 mins from LPS treatment, effect blocked by JSH-23. Draq5 was used as nuclear stain (in red). Magnification 60X. D) NF-κB transcription factor inhibition with JSH-23 do not alter H_2O_2 CD80 up regulation. CD80 expression was measured by flow cytometry and quantitative data expressing not significant changes of CD80 expression after H_2O_2 treatment. Data are representative of two independent experiments (n=4).
The ordinary one-way ANOVA followed by unpaired T test with equal SD were used for comparisons.

5.2.2 DNA damage/p53 signalling is not required for the induction of CD80 by ROS

One of the most important tumour suppressors is p53 (Trp53 in mice), which responds to various stresses, such as DNA damage, oncogene activation, hypoxia including excess of free radicals production that lead to genomic instability [204,209].

H_2O_2 is known to provoke an appearance of both single- and double- strand breaks that can trigger DDR (DNA damage response). Generally, DDR is characterized by activation of ataxia-telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), and ɣH2AX is recognized as a reliable marker of DDR. The activation of the previously described kinases (ATM/ATR) usually trigger p53 transcription factor activation [210–213] (Fig 23). Thus, it is possible that the H_2O_2 – CD80 up regulation could be mediated through the activation of the DNA damage signalling.

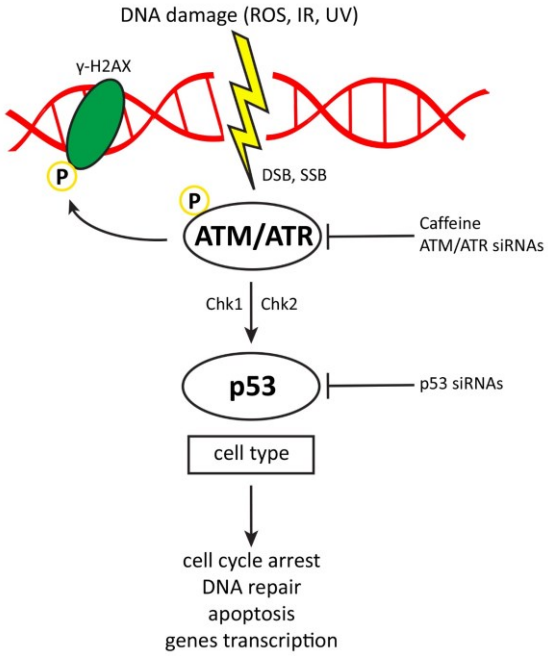


Fig. 23. *DNA damage/p53 signalling.*
Adapted from: Amanda K. Ashley & Christopher J. Kemp (2018), DNA-PK, ATM, and ATR: Pikking on p53, Cell Cycle, 17:3, 275-276, DOI: 10.1080/15384101.2017.1412147 [214] .

We showed that induction of ɣH2AX occurs with H_2O_2 treatment in CT26 cell line. This induction was completely blocked by caffeine, a known inhibitor of ATM/ATR pathway (Fig. 24A). Remarkably, flow cytometry results indicated that caffeine effectively decreased H_2O_2-induced CD80 expression in CT26 colon cancer cells, revealing that DNA damage response could be effectively required for the up regulation of CD80 (Fig. 24B).

To further validate this conclusion, we tested CD80 expression upon depletion of ATM/ATR by siRNA in CT26 cells. The efficiency of knockdown was confirmed by RT-PCR

but, as shown in Fig. 24C, ATM/ATR knockdown failed to prevent H_2O_2 induced CD80 up-regulation.

Overall, the results of experiments with caffeine and ATM/ATR siRNAs although suggest Trp53 as final actor of the DNA damage signalling are quite contradictory, therefore we set up further experiments to elucidate the role of this pathway in H_2O_2-induced CD80 expression.

First, we choose to evaluate if the cyclin-dependent kinase inhibitor 1A (Cdkn1a; encoding p21), once of most known p53 target genes, was over expressed in CT26 after treatment with H_2O_2. Data obtain from our qPCR underlined that there was not p21 transcription up-regulation (Fig. 24D). As positive control for the up regulation of p21 we used nutlin3a, a small molecule known to activate p53, and qPCR underlined that there was a significant up regulation of p21 transcription in CT26 cell line (data not shown).

To confirm the exclusion of Trp53 we assessed the knockdown of p53 by siRNA. The silencing assays confirmed data obtain in RT-pcr with p21, the p53 silencing prevent only in a minimal part the CD80 up regulation induced by free radicals as detected by flow cytometry (Fig. 24E). The Trp53 silencing was confirmed with qPCR.

Taken together, our data show that CD80 overexpression induced by ROS is not DNA damage/Trp53 signalling dependent and the result obtained with the use of caffeine were most likely due to its capacity in decreasing intracellular reactive oxygen species [215,216] as demonstrated by immunofluorescence assay with the fluorogenic dye CM-H_2DCFDA (Fig. 24F).

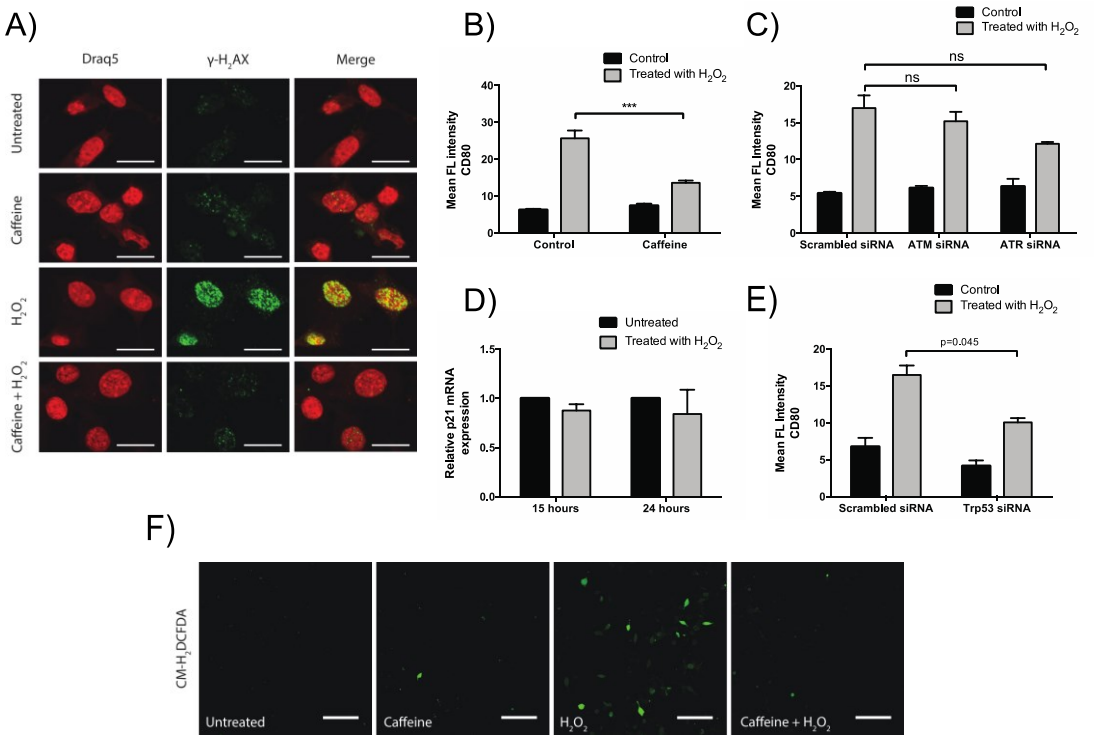


Fig. 24. *Induction of CD80 expression mediated by ROS do not relies on DNA damage/p53 pathway in CT26 cell line.*

A) H_2O_2 increased γH2AX foci formation in cells. Representative confocal images showing increase in γH2AX foci generation (green fluorescence) following 30 mins from H_2O_2 treatment, induction completely blocked by caffeine. Draq5 was used as nuclear stain (in red). Magnification 60X. B) Treatments with caffeine significantly suppressed the positive effect of H_2O_2 on the CD80 up regulation, CD80 expression was measured by flow cytometry, quantitative data expressing changes of CD80 expression after H_2O_2 and caffeine treatments. Data are representative of three independent experiments (n=6). C) Depletion of ATM/ATR by siRNA in CT26 cells did not prevent H_2O_2 induced CD80 up-regulation as measured by flow cytometry analysis. Quantitative data expressing changes of CD80 expression after H_2O_2 and siRNAs treatments. Data are representative of two independent experiments (n=2). D) Relative p21 mRNA expression measured by RT-PCR after 15 and 24 hours H_2O_2 treatment expressed as fold change using 18s as endogenous control. Data represent mean ± SEM (n= 2-4) and are representative of two independent experiment. E) Knockdown of p53 by siRNA preserved only in minimal part the CD80 up regulation induced by free radicals as detected by flow cytometry analysis (CD80 mean fluorescence intensity). Data are representative of two independent experiments (n=2). F) Representative confocal images showing increase in ROS generation (CM-H_2DCFDA green fluorescence) following overnight H_2O_2 treatment. Treatment with caffeine suppressed the positive effect of H_2O_2. Magnification 40X.

The ordinary one-way ANOVA followed by unpaired T test with equal SD were used for comparisons.

5.2.3 Oxidative stress mediated CD80 induction relies on MAPK activation through STAT3 transcription factor

Two of the major mitogen-activated protein kinase (MAPK) pathways, c-Jun-N-terminal kinases (JNKs) and p38, are known to be activated by a variety of stimuli including oxidative stress [217–219].

Thus, to investigate the role of these kinases we suppressed their activity by using two selective pharmacologic MAPKs inhibitors, for each kinase, with unrelated chemical structure for each kinase (SB203580 and BIRB796 for p38, SP600125 and AS601245 for JNK). All the inhibitors partially prevented H_2O_2 induced CD80 up regulation, demonstrating that both p38 and JNK are involved in CD80 induction (Fig. 25A).

STAT proteins are known to be highly expressed in different types of cancers and play a crucial role in cancer inflammation and immunosuppression. Moreover, activation of STAT3 may occur via phosphorylation by MAPKs [220–222] suggesting the involvement of STAT3 as transcription factor of CD80 induced by ROS.

Since the activation of the transcription factor STAT3 can occur via phosphorylation by MAPK, we checked its status in CT26 upon H_2O_2 treatment. Notably, we observed that oxidative stress activated STAT3, as shown by increased levels of phosphorylation on Tyr705 (Fig. 25B and 25C). Thus, we tested MAPKs inhibitor effect on STAT3 phosphorylation. As shown in Fig. 25D, oxidative stress mediated STAT3 activation was abolished by CEC treatment with SB203580, BIRB 796 and AS601245 inhibitors. Moreover, pharmacological inhibition of STAT3 using 5,1-DPP or its knockdown by siRNA in CT26 cell line significantly decreased CD80 expression induced by free radicals (Fig. 25E and 25F). Altogether, these data suggest that ROS induce CD80 expression via MAPK pathways that activate STAT3 in colon epithelial cells.

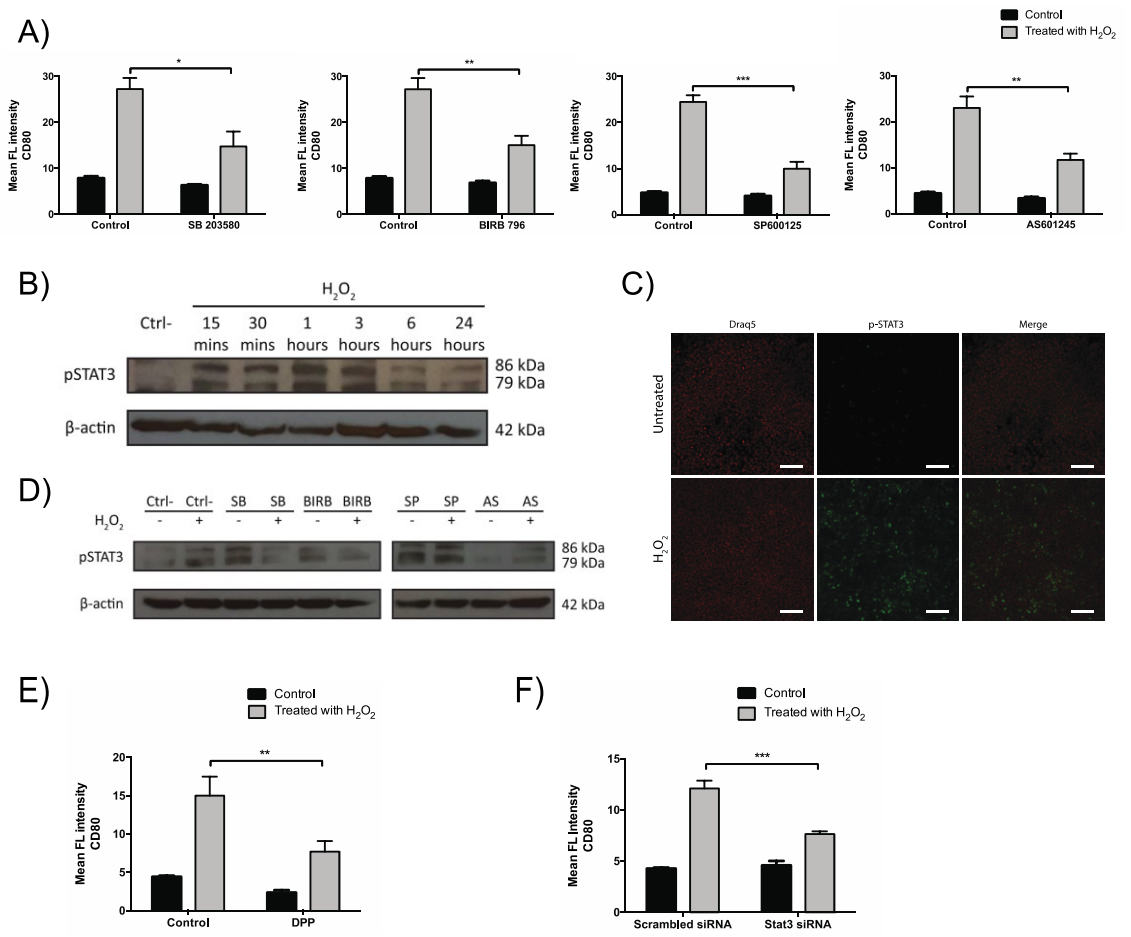


Fig. 25. *Induction of CD80 expression mediated by ROS relies on MAPK activation through STAT3 transcription factor in CT26 cell line.*

A) Treatments with MAPKs inhibitors (SB203580, BIRB 796 for p38 and SP600125, AS601245 for JNKs partially suppressed the positive effect of H_2O_2 induced CD80-up regulation. CD80 expression was measured by flow cytometry, quantitative data expressing changes of CD80 expression after the different treatments. Data are representative of three independent experiments (n=6). B) H_2O_2 - induced phospho-STAT3 phosphorylation tested by Western Blotting. β-actin was used as loading control. C) H_2O_2 - induced phospho-STAT3 phosphorylation tested by immunofluorescence assay. Representative confocal images showing increase in phospho-STAT3 (green fluorescence) following one hour from H_2O_2 treatment. Draq5 was used as nuclear stain (in red). Magnification 20X. D) H_2O_2 - induced phospho-STAT3 phosphorylation inhibition by MAPKs inhibitors tested by Western Blotting. β-actin was used as loading control. E) Treatments with DPP (STAT3 pharmacological inhibitor) significantly suppressed the positive effect of H_2O_2 on the CD80 up regulation as measured by flow cytometry. Quantitative data expressing changes of CD80 expression after H_2O_2 and DPP treatments. Data are representative of four independent experiments (n=8). F) Knockdown of STAT3 by siRNA prevented CD80 up regulation induced by H_2O_2 as detected by flow cytometry. Quantitative data expressing changes of CD80 expression after H_2O_2 and DPP treatments. Data are representative of three independent experiments (n=5). The ordinary one-way ANOVA followed by unpaired T test with equal SD were used for comparisons.

5.3 TLR4 signalling reduces AOM – induced colonic tumorigenesis in mice

To better understand the role of TLR4 receptor in sporadic colon cancer we used the previously described model with the mutagenic agent azoxymethane AOM, which has been shown to induce colonic tumours in mice. C57BL/6 mice were used as Wild Type and were the genetic background for our TLR4^{KO} animals.

The incidence and extension of high- and low-grade dysplasia was comparable between WT and TLR4^{KO} mice after 6 and 8 months following AOM treatment (Fig.26A). However, macroscopic colonic polyps in TLR4^{KO} mice were significantly more abundant as compared to the wild type strain (Fig. 26C). Moreover, invasive colonic carcinoma was significantly more frequent in TLR4^{KO} mice compared to Wild type mice sacrificed after 8 months from the first AOM injection (Fig. 26B).

Overall, these data reveal a protective role of TLR4 derived signals in sporadic colon tumorigenesis *in vivo*.

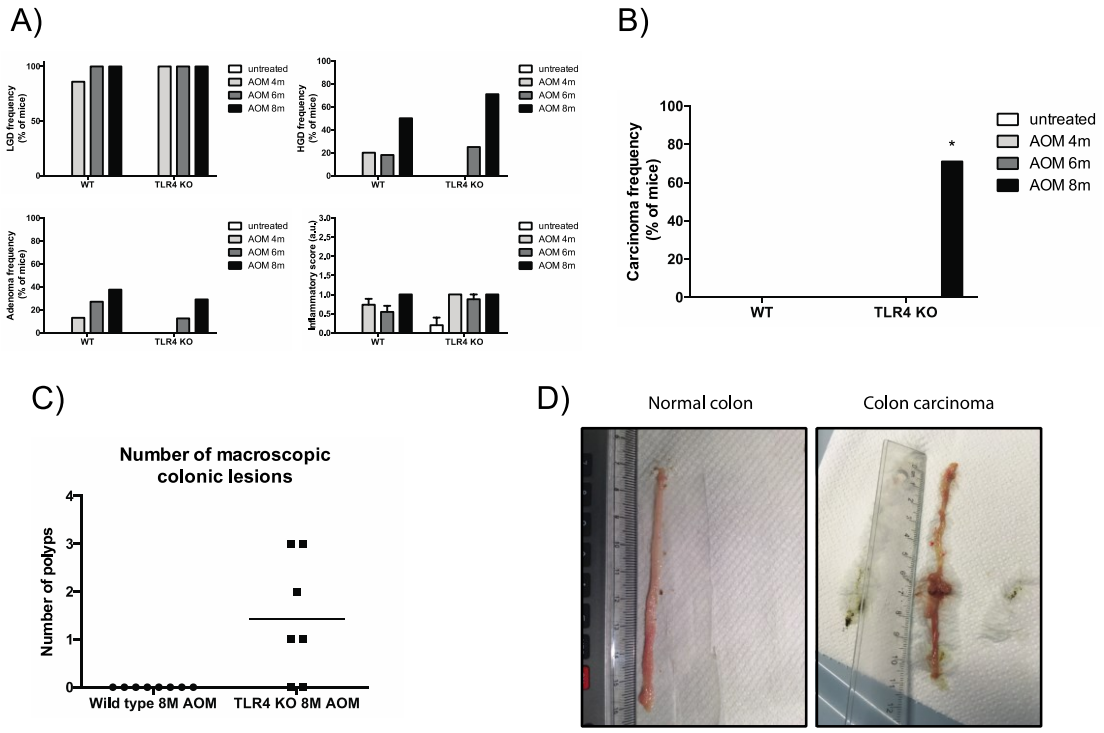


Fig. 26. *TLR4 controls the progression of colonic neoplastic lesions in AOM carcinogenesis model. A) Frequency of adenoma, high grade dysplasia (HGD) and low-grade dysplasia (LGD) in AOM-treated WT and TLR4^{KO} mice euthanized 4, 6 and 8 months after the first AOM injection. B) Frequency of carcinoma in AOM-treated WT and TLR4^{KO} mice euthanized 4, 6 and 8 months after*

the first AOM injection. C) Number of macroscopic lesions detected in TLR4^{KO} mice treated with AOM and representative images of a normal colon VS a colon showing visible polyps.
The Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for comparisons (n=7-15 mice per group).

5.4 Less efficient presentation of tumour associated antigens in TLR4 deficient mice

As previously described, antitumor immune responses are mainly mediated by CD8$^+$ cytotoxic T lymphocytes and CD4$^+$ helper T lymphocytes that recognize amino acids derived from TAAs bound by MHC class I molecules on tumour cells or by MHC II molecules on professional APCs, respectively.

Flow cytometric analysis of cytokeratin positive colonic epithelial cells isolated from WT mice 8 months after AOM treatment, revealed that the expression of MHC class I and MHC class II remained substantially stable. On the contrary, in colonic epithelial cells of AOM-treated TLR4^{KO} mice a significant reduction of MHC class I and class II was evident, suggesting a less efficient presentation of the tumoral antigens by the TLR4 deficient strain (Fig. 27A) Concomitant with the decrease of the MHC expression, in 8 months AOM-treated TLR4^{KO} mice there was also a significant deficiency of activated lymphocytes in the colonic mucosa, both CD8+ (CD28+ and CD38+) and CD4+ (CD25+) (Fig. 27B).

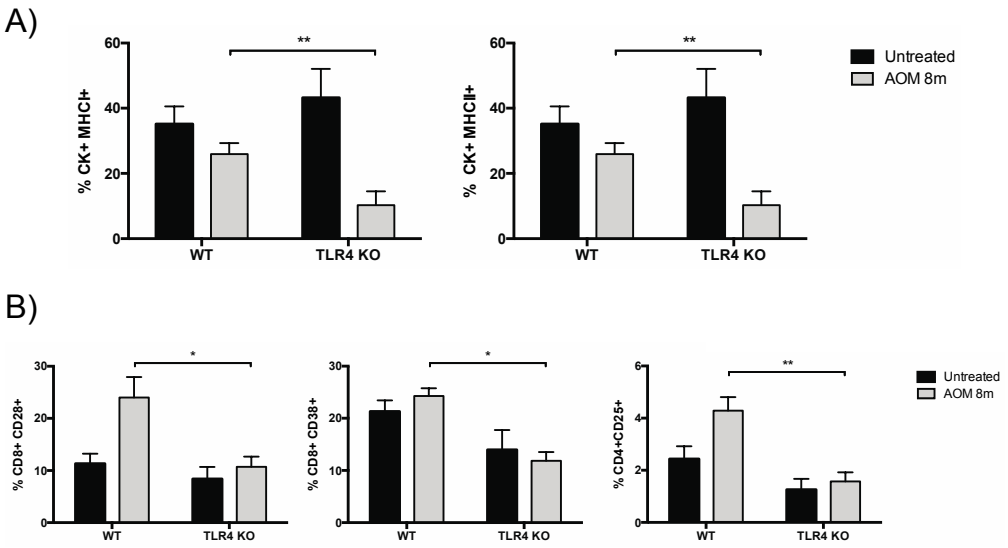


Fig. 27. *Less efficient presentation of the tumoral antigen by the TLR4^{KO} mice.*

A) Flow cytometric analysis of intestinal epithelial cells isolated from the colon of WT and TLR4^{KO} mice, treated or not with AOM and sacrificed 8 months after the first AOM injection. Quantitative data expressing changes in the percentage of MHC class I and II expression on intestinal epithelial cells (pan-cytokeratin+). B) Quantitative data expressing changes in the percentage of CD8+CD28+ and CD8+CD38 lymphocytes from the colons of WT and TLR4^{KO} mice, treated or not with AOM and sacrificed 8 months after the first AOM injection, measured by flow cytometry. The Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for comparisons (n=5-13 mice per group).

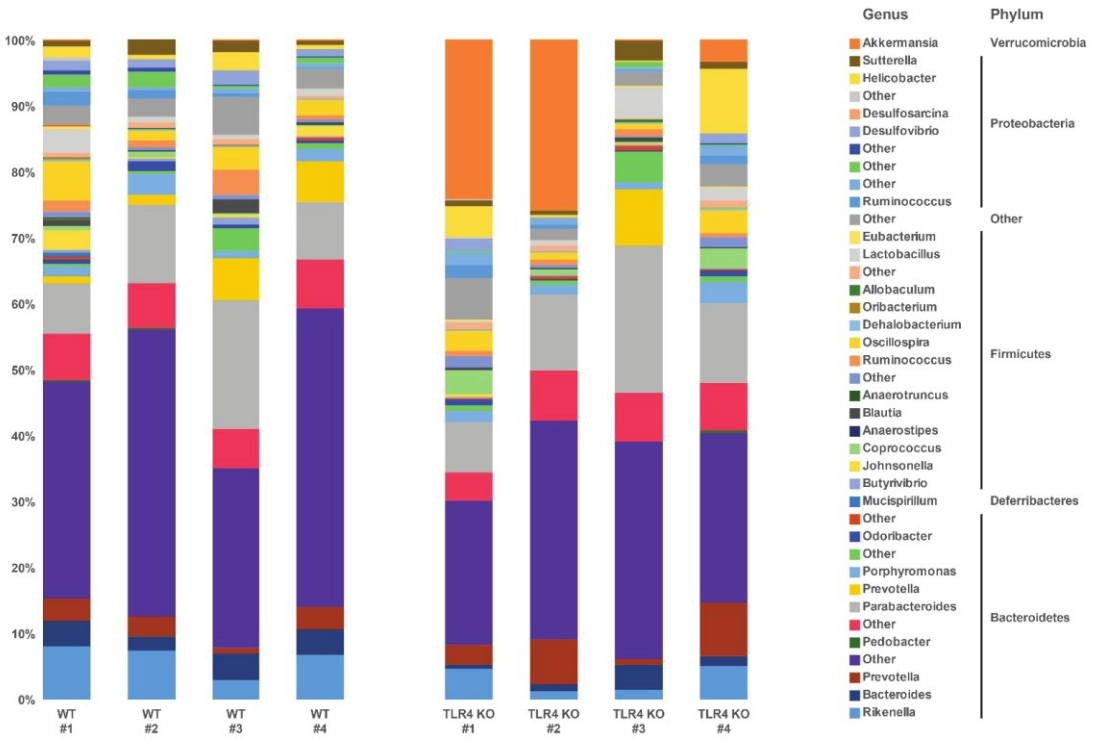
5.5 Lack of TLR4 does not result in significant changes in the composition of intestinal microbiota

It has been demonstrated that microbiota has a key role to modulate immune cells activities [223]. We therefore compared the composition of gut microbiota of WT and TLR4 deficient mice.

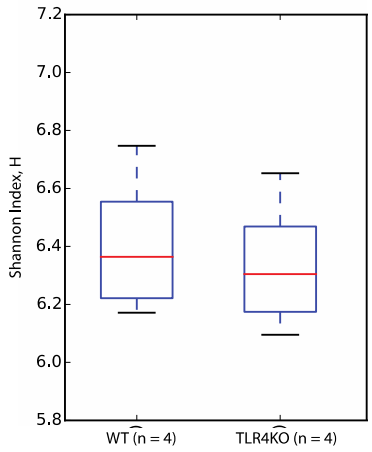
To this goal it was performed a 16S rDNA sequencing of faecal samples. Accordingly to previous studies [224], most faecal bacteria belong to the Bacteroidetes and Firmicutes phyla. Moreover, WT and TLR4 deficient mice display a comparable composition of the microbiota (Fig 28A). To identify bacterial taxa that are significantly affected by TLR signalling, we used the Student's *t* test to compare the relative number of specific taxa colonizing KO and WT mice. Taxa of low abundance (less than 100 counts in all mice) were not included in this analysis. Furthermore, to establish whether TLR signalling has an impact on the diversity of the intestinal microbiota, the Shannon diversity index was considered for each sample. No differences in the Shannon diversity index were found between TLR4KO and WT mice (Fig. 28B).

Additionally, we analysed bacterial SCFAs, an important class of molecules that act as link between the microbiota and the immune system and whose composition reflect the composition of gut microbiota[225]. As shown in Fig. 28C, the level of the most important SCFAs do not show significant difference between the two genotypes.

A)



B)



C)

Mice	Short-chain fatty acids composition (µmol/g)		
	Butyric acid	Propionic acid	Acetic acid
Wild Type	4,28 ± 1,60	2,65 ±0,70	8,56 ± 2,72
TLR4^{KO}	3,14 ± 0,90	5,08 ± 1,04	12,94 ± 2,52

Fig. 28. *Impact of TLR4 signalling on the bacterial composition of the intestinal microbiota.*
A) Phylogenetic classification of 16S rDNA frequencies in the faeces from TLR4^{KO} mice and WT mice. Each bar represents the mean of the microbiota composition of the single mice. The most predominant bacterial taxa are shown and labelled with different colours as indicated. Bacterial taxa were obtained by classification of 16s rDNA sequences to the genus level using Illumina. B) Shannon diversity index calculated for each sample. C) Quantification of SCFAs levels in faecal samples from WT and TLR4^{KO} mice by GC-MS.
To identify bacterial taxa that were significantly affected by TLR4 signalling, we used the Student's t test to compare the relative number of specific taxa colonizing KO and WT mice.

5.5.1 TLR4 deficient mice showed a decreased level of mature DCs critical for antitumor T cell priming

We next attempted to elucidate whether a functional deficit of DCs in TLR4s could explain the lack of T cell activation. To address this issue, WT and TLR4^{KO} DCs were stimulated for 24 hours with LPS. As opposed to WT-derived DCs, TLR4^{KO} DCs failed to up-regulate CD80 in response to LPS (Fig. 29A).

Moreover, to determine the relevance of TLR4-derived signals in DCs maturation, we evaluated the expression of specific molecular markers (i.e. XCR1 and Batf3) of DCs development within the carcinoma microenvironment in the colonic mucosa. As shown in Fig. 29B, both XCR1, a chemokine receptor exclusively expressed by DCs, and Batf3, a transcription factor preferentially expressed by DCs, mRNA were expressed at significant lower levels in TLR4^{KO} as compared to WT mice.

Overall, our data suggest that TLR4 deficient mice showed a diminished level of mature DCs fundamental for antitumor T cell priming.

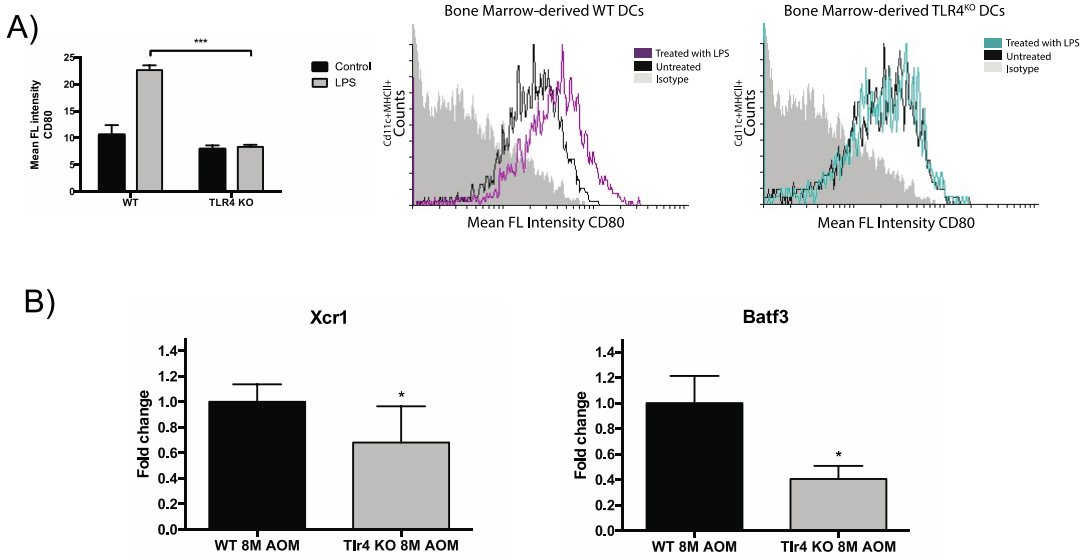


Fig. 29. *TLR4KO mice showed a decreased level of mature DCs critical for antitumor T cell priming.*
A) WT and TLR4KO bone marrow-derived DCs were stimulated for 24 hours with LPS and only WT bone marrow-derived DCs showed a response as measured by flow cytometry. Data are representative of three independent experiments (n=6). The ordinary one-way ANOVA followed by unpaired T test with equal SD were used for comparisons. Filled histogram correspond to an isotype control, continuous black line corresponds to untreated DCs, continuous violet line corresponds to WT DCs treated with LPS and continuous blue line corresponds to TLR4KO DCs

treated with LPS, respectively. B) Batf3 and XCR1 mRNA expression were assessed by RT-PCR analysis on colonic mucosa. The results are expressed as fold change using 18s as endogenous control. Results are shown as mean ± SEM of two independent experiment (n=7). The Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for comparisons.

6 Conclusions and discussion

In a contest in which the 5-year survival rate for patients with metastatic CRC is less than 10%, immunotherapy may represent an effective new therapeutic approach for treating CRC patients and/or preventing relapse [133]. Cancer immunosurveillance is a critical step that the tumour has to overcome in order to develop in the organism before it becomes clinically apparent, thus the understanding of the effector molecules involved in this mechanism is fundamental to improve new immunotherapeutic approaches, even in the early stages of carcinogenesis. In the current thesis, I've studied the role of CD80 co-stimulatory molecule expression in colonic epithelial cells in the progression of sporadic colorectal carcinogenesis.

As previously described, CD80 is over-expressed by epithelial cells in human colonic pre-neoplastic lesions compared to normal and tumour CEC suggesting that the expression of this co-stimulatory molecule on dysplastic epithelial cells is crucial in successful immune surveillance mechanisms of sporadic colorectal cancer. To confirm this hypothesis, we used the AOM model that generating reactive metabolites is responsible for DNA methylation also in epithelial cells. The data obtained in the animal model confirmed the results observed in human preneoplastic lesions supporting the possible pivotal role of CD80 at the beginning of CRC progression. Additionally, we observed that lack of functional CD80 in colonic mucosa (with the use of CD80 neutralizing antibody and $CD80^{KO}$ mice) accelerates the progression of colonic carcinogenesis probably through a failure to activate lymphocites and NK response against emerging dysplastic epithelial cells [226–229]. Furthermore, data obtained from the use of CD80KO/WT bone marrow chimeras in the experimental model support that CD80 epithelial expression contribute to inhibition of dysplasia development.

As stated before, CD80 is usually expressed by professional APC and little is known about CD80 expression and modulation in non-hematopoietic cells. Anyway, few reports suggest that CD80 expression could be induced under stress conditions [230–232]. According with this, it is also known that AOM causes pathological changes in the colonic mucosa by increasing oxidative stress and consequently genotoxicity. This is in line with the results obtained in the *in vivo* model in which was confirmed that AOM-treated colonic

mucosa was characterized by an oxidative microenvironment. So, we recreated the same condition *in vitro* verifying that pro-oxidant agent H_2O_2 was able to generate oxidative stress in CT26 cells. The *in vitro* experiment showed also a strong up-regulation of CD80 in CT26 cells and primary IECs under oxidative stress. Overall, data described so far reflect the ability of cellular ROS to mediate relevant biological responses. Indeed, it is known that low ROS levels are involved in cellular homeostasis, but in conditions of imbalance between production and detoxification, ROS might act as pro-carcinogens. Radicals species mainly derived from innate immune cells and mucosa-resident cells [73,74] together with the contribution of risk factors like alcohol and smoking, known to be involved in colonic carcinogenesis [75,76].

Next, we analysed which pathways could be involved in oxidative stress mediated CD80 upregulation in CEC and we demonstrated that CD80 expression in CT26 cells relies on two different MAPK pathways (p38MAPKs and JNKs) through the activation of the transcription factor STAT3 (Fig. 30). Interestingly, CD80 induction by oxidative stress was not a consequence of apoptosis, NF-κB signalling or ATM/ATR pathway/Trp53 activation, as proved by different tests. The role of STAT3 in colon carcinogenesis is still controversial. Active STAT3 is often found at the invasive edge of different tumours and adjacent to inflammatory cells suggesting a relevant role in the crosstalk between immune system e tumour cells [233]. In particular, mice lacking STAT3 in IECs are more resistant to the onset of AOM-DSS induced colon carcinoma [234] but ablation of STAT3 in IECs of APC^{Min} although reducing early adenomas multiplicity promotes tumour progression at later stages, leading to invasive carcinomas [235]. Finally, in a recent study, STAT3 depletion in two wnt/b-catenin-dependent models of sporadic intestinal tumorigenesis revealed a complex intracellular process in IECs that enhances the induction of a CD8+ T cell based adaptive immune response thorough an elevated mitophagy [236]. Therefore, STAT3 seems to acquire specific functions according to the different tumoral milieu, however, further investigation is needed to explore this hypothesis.

In conclusion, we highlighted the complex regulation of CD80 molecule in CECs that proves to have an important role as mediator of immune defence in the early stages of colon cancer development.

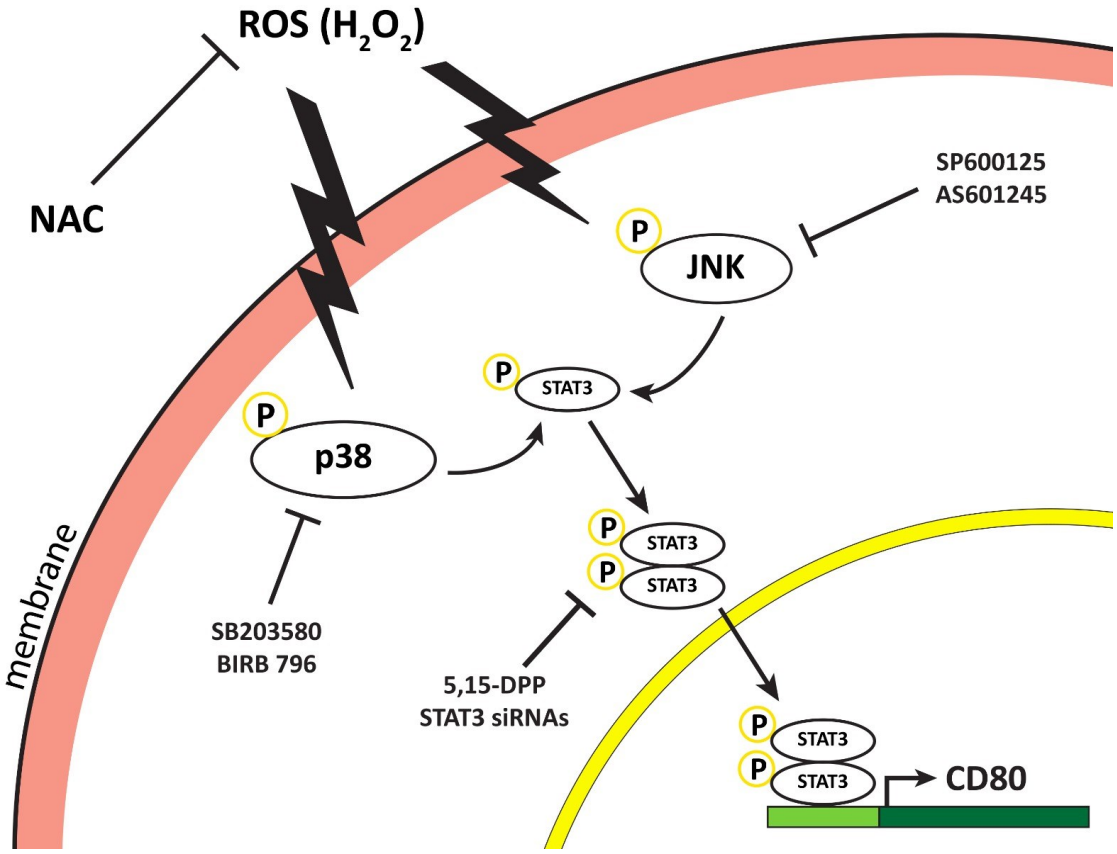


Fig. 30. *A pathway interaction scheme displaying the proposed molecular mechanism of CD80 up-regulation in intestinal epithelial cells under oxidative stress.*

As well, while CRC was almost totally a public health issue in industrialized countries up to few decades ago, actually it is also a growing problem in emerging countries due to the adoption of a westernized lifestyle, increased consumption of red meat and high-calories diets. A number of studies have suggested that the microbiota composition could be affected by these changes, having a fundamental role in intestinal colon cancer carcinogenesis [237,238]. A large body of evidences supports a relationship between infective agents and human CRC acting through different mechanisms such as production of DNA damaging superoxide radicals, production of genotoxins, T helper cell-dependent induction of cell proliferation and Toll-like receptor mediated induction of pro-carcinogenic pathways [239]. In particular, TLR signalling has been implicated in the inflammatory responses in intestinal epithelial cells (IECs). Thus, in a several studies investigating tumour growth, survival and progression reported that TLR4 signalling activated by LPS was strongly linked to inflammation [203,240,241]. However, its role remain still controversial in non-inflammatory carcinogenesis. Different findings support the

potential role of the TLR4 signalling in intestinal tumour development [242–244], on the other hand potent anticancer effects have been illustrated with the administration of TLR4 agonists [204,245,246]. Therefore, the role of TLR4 pathway in CRC tumour development is strongly dependent on the milieu and deserves further investigation. To contribute to clarify this point, the same experimental model of sporadic colorectal carcinogenesis with AOM was used. The significant increase of the carcinoma frequency in $TLR4^{KO}$ mice compared to WT mice revealed a protective role of TLR4 in sporadic colon tumorigenesis *in vivo*. This data was in line with the results obtained analysing the immune response against tumour cells in the progression phases that was significantly diminished in $TLR4^{KO}$ mice, suggesting a less effective presentation of the tumoral antigens in this strain.

Nevertheless, it remained possible that the composition of the microbiota might be affected promoting the tumour growth [247,248] as a consequence of the TLR4 signalling absence. To this end, the relative representation of different bacterial taxa and the SCFAs composition (bacterial fermentation products that reflect microbiota structure) in faeces samples were verified. Our results showed that TLR4 deficit does not result in significant changes in the composition of the microbiota excluding its direct role on tumor progression in the experimental model.

It is known that the lack of TLR4 signalling might generate inconsistent signals to mucosal immune cells [249,250]. Indeed, *in vitro* experiments with bone marrow derived DCs confirmed the necessary requirement of TLR4 pathway to obtain mature and competent DCs.

In conclusion, this work revealed that TLR4 signalling is protective in sporadic colorectal carcinogenesis through its crucial role in the immune response activation against tumour cells. Thus, our data support that TLR4 signalling is not certainly associated with tumour growth and progression, but other regulatory processes enhanced by TLR4 stimulation should be considered when the impact of microbiota on tumour is evaluated.

7 References

1. Paranzini Bordini, H. *et al.* Protective effect of metformin in an aberrant crypt foci model induced by 1,2-dimethylhydrazine: Modulation of oxidative stress and inflammatory process. *Mol. Carcinog.* (2016). doi:10.1002/mc.22545

2. Torre, L. A. *et al.* Global cancer statistics, 2012. *CA. Cancer J. Clin.* **65**, 87–108 (2015).

3. Ferlay, J. *et al.* Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012: Globocan 2012. *Int. J. Cancer* **136**, E359–E386 (2015).

4. Rosa, B. Effectiveness and safety of monoclonal antibodies for metastatic colorectal cancer treatment: systematic review and meta-analysis. *ecancermedicalscience* **9**, (2015).

5. Van Cutsem, E., Nordlinger, B., Cervantes, A. & ESMO Guidelines Working Group. Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **21 Suppl 5**, v93-97 (2010).

6. Jemal, A. *et al.* Global cancer statistics. *CA. Cancer J. Clin.* **61**, 69–90 (2011).

7. Roper, J. & Hung, K. E. Molecular Mechanisms of Colorectal Carcinogenesis. in *Molecular Pathogenesis of Colorectal Cancer* (ed. Haigis, Ph.D., K. M.) 25–65 (Springer New York, 2013).

8. Frank, S. A. *Dynamics of cancer: incidence, inheritance, and evolution*. (Princeton University Press, 2007).

9. Pancione, M. *et al.* Genetic and Epigenetic Events Generate Multiple Pathways in Colorectal Cancer Progression, Genetic and Epigenetic Events Generate Multiple Pathways in Colorectal Cancer Progression. *Pathol. Res. Int. Pathol. Res. Int.* **2012**, **2012**, e509348 (2012).

10. Joslyn, G., Richardson, D. S., White, R. & Alber, T. Dimer formation by an N-terminal coiled coil in the APC protein. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11109–11113

(1993).

11. Goss, K. H. & Groden, J. Biology of the adenomatous polyposis coli tumor suppressor. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **18**, 1967–1979 (2000).

12. Sieber, O. M., Tomlinson, I. P. & Lamlum, H. The adenomatous polyposis coli (APC) tumour suppressor--genetics, function and disease. *Mol. Med. Today* **6**, 462–469 (2000).

13. Kaplan, K. B. *et al.* A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat. Cell Biol.* **3**, 429–432 (2001).

14. van Es, J. H., Giles, R. H. & Clevers, H. C. The many faces of the tumor suppressor gene APC. *Exp. Cell Res.* **264**, 126–134 (2001).

15. Lamlum, H. *et al.* APC mutations are sufficient for the growth of early colorectal adenomas. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2225–2228 (2000).

16. Sjöblom, T. *et al.* The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268–274 (2006).

17. Fearon, E. R. Molecular genetics of colorectal cancer. *Annu. Rev. Pathol.* **6**, 479–507 (2011).

18. Network, T. C. G. A. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330–337 (2012).

19. Albuquerque, C. *et al.* The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. *Hum. Mol. Genet.* **11**, 1549–1560 (2002).

20. March, H. N. *et al.* Insertional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. *Nat. Genet.* **43**, 1202–1209 (2011).

21. van der Flier, L. G. & Clevers, H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu. Rev. Physiol.* **71**, 241–260 (2009).

22. Lau, T. *et al.* A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. *Cancer Res.* **73**, 3132–3144 (2013).

23. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer.

Cell **87**, 159–170 (1996).

24. Knudson, A. G. Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10914–10921 (1993).

25. Dienstmann, R. *et al.* Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat. Rev. Cancer* **17**, nrc.2016.126 (2017).

26. Hedrick, L. *et al.* The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev.* **8**, 1174–1183 (1994).

27. Fearon, E. R. *et al.* Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* **247**, 49–56 (1990).

28. Thiagalingam, S. *et al.* Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat. Genet.* **13**, 343–346 (1996).

29. Zhou, S. *et al.* Targeted deletion of Smad4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2412–2416 (1998).

30. Walther, A. *et al.* Genetic prognostic and predictive markers in colorectal cancer. *Nat. Rev. Cancer* **9**, 489–499 (2009).

31. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. p53 mutations in human cancers. *Science* **253**, 49–53 (1991).

32. Rodrigues, N. R. *et al.* p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7555–7559 (1990).

33. López, I. *et al.* Different mutation profiles associated to P53 accumulation in colorectal cancer. *Gene* **499**, 81–87 (2012).

34. Mojarad, E. N., Kuppen, P. J., Aghdaei, H. A. & Zali, M. R. The CpG island methylator phenotype (CIMP) in colorectal cancer. *Gastroenterol. Hepatol. Bed Bench* **6**, 120–128 (2013).

35. Pino, M. S. & Chung, D. C. THE CHROMOSOMAL INSTABILITY PATHWAY IN COLON CANCER. *Gastroenterology* **138**, 2059–2072 (2010).

36. McIntyre, R. E., Buczacki, S. J. A., Arends, M. J. & Adams, D. J. Mouse

models of colorectal cancer as preclinical models. *BioEssays* **37**, 909–920 (2015).

37. Kuismanen, S. A., Holmberg, M. T., Salovaara, R., de la Chapelle, A. & Peltomäki, P. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. *Am. J. Pathol.* **156**, 1773–1779 (2000).

38. Miyakura, Y. *et al.* Extensive but hemiallelic methylation of the hMLH1 promoter region in early-onset sporadic colon cancers with microsatellite instability. *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.* **2**, 147–156 (2004).

39. Herman, J. G. & Baylin, S. B. Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042–2054 (2003).

40. Boland, C. R. & Goel, A. Microsatellite Instability in Colorectal Cancer. *Gastroenterology* **138**, 2073-2087.e3 (2010).

41. Fearnhead, N. S., Wilding, J. L. & Bodmer, W. F. Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis. *Br. Med. Bull.* **64**, 27–43 (2002).

42. Guinney, J. *et al.* The consensus molecular subtypes of colorectal cancer. *Nat. Med.* **21**, 1350–1356 (2015).

43. Scarpa, M. *et al.* CD80-CD28 signaling controls the progression of inflammatory colorectal carcinogenesis. *Oncotarget* **5**, (2015).

44. Jess, T., Rungoe, C. & Peyrin-Biroulet, L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.* **10**, 639–645 (2012).

45. Eaden, J. A., Abrams, K. R. & Mayberry, J. F. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* **48**, 526–535 (2001).

46. Lakatos, P.-L. Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies. *World J. Gastroenterol.* **14**, 3937 (2008).

47. Scarpa, M. Inflammatory colonic carcinogenesis: A review on pathogenesis and immunosurveillance mechanisms in ulcerative colitis. *World J.*

Gastroenterol. **20**, 6774 (2014).

48. Scarpa, M. *et al.* Mucosal immune environment in colonic carcinogenesis: CD80 expression is associated to oxidative DNA damage and TLR4–NFκB signalling. *Eur. J. Cancer* **49**, 254–263 (2013).

49. Ribeiro, M. B. *et al.* Colorectal adenocarcinoma in Crohn's disease. *Ann. Surg.* **223**, 186–193 (1996).

50. Ahmed, F. E. Effect of diet, life style, and other environmental/chemopreventive factors on colorectal cancer development, and assessment of the risks. *J. Environ. Sci. Health Part C Environ. Carcinog. Ecotoxicol. Rev.* **22**, 91–147 (2004).

51. McKeown-Eyssen, G. E. & Bright-See, E. Dietary factors in colon cancer: international relationships. *Nutr. Cancer* **6**, 160–170 (1984).

52. Liu, K. *et al.* Dietary cholesterol, fat, and fibre, and colon-cancer mortality. An analysis of international data. *Lancet Lond. Engl.* **2**, 782–785 (1979).

53. Armstrong, B. & Doll, R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int. J. Cancer* **15**, 617–631 (1975).

54. Bingham, S. A. *et al.* Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet Lond. Engl.* **361**, 1496–1501 (2003).

55. Ishikawa, H. *et al.* Randomized trial of dietary fiber and Lactobacillus casei administration for prevention of colorectal tumors. *Int. J. Cancer* **116**, 762–767 (2005).

56. Schatzkin, A. *et al.* Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group. *N. Engl. J. Med.* **342**, 1149–1155 (2000).

57. Jacobs, E. T. *et al.* Fiber, sex, and colorectal adenoma: results of a pooled analysis. *Am. J. Clin. Nutr.* **83**, 343–349 (2006).

58. Sinha, R. *et al.* Well-done, grilled red meat increases the risk of colorectal

adenomas. *Cancer Res.* **59**, 4320–4324 (1999).

59. Lee, D.-H., Anderson, K. E., Folsom, A. R. & Jacobs, D. R. Heme iron, zinc and upper digestive tract cancer: the Iowa Women's Health Study. *Int. J. Cancer* **117**, 643–647 (2005).

60. Oates, P.-S. & West, A.-R. Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. *World J. Gastroenterol.* **12**, 4281–4295 (2006).

61. Limsui, D. *et al.* Cigarette Smoking and Colorectal Cancer Risk by Molecularly Defined Subtypes. *JNCI J. Natl. Cancer Inst.* **102**, 1012–1022 (2010).

62. Terry, P. D., Miller, A. B. & Rohan, T. E. Prospective cohort study of cigarette smoking and colorectal cancer risk in women. *Int. J. Cancer* **99**, 480–483 (2002).

63. Tsong, W. H. *et al.* Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese Health Study. *Br. J. Cancer* **96**, 821–827 (2007).

64. Ferrari, P. *et al.* Lifetime and baseline alcohol intake and risk of colon and rectal cancers in the European prospective investigation into cancer and nutrition (EPIC). *Int. J. Cancer* **121**, 2065–2072 (2007).

65. Pöschl, G. & Seitz, H. K. Alcohol and cancer. *Alcohol Alcohol. Oxf. Oxfs.* **39**, 155–165 (2004).

66. Baan, R. *et al.* Carcinogenicity of alcoholic beverages. *Lancet Oncol.* **8**, 292–293 (2007).

67. Kanai, Y. & Hirohashi, S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. *Carcinogenesis* **28**, 2434–2442 (2007).

68. Weinert, B. T. & Timiras, P. S. Invited review: Theories of aging. *J. Appl. Physiol. Bethesda Md 1985* **95**, 1706–1716 (2003).

69. White, E., Jacobs, E. J. & Daling, J. R. Physical activity in relation to colon cancer in middle-aged men and women. *Am. J. Epidemiol.* **144**, 42–50 (1996).

70. Friedenreich, C. M. & Orenstein, M. R. Physical Activity and Cancer

Prevention: Etiologic Evidence and Biological Mechanisms. *J. Nutr.* **132**, 3456S-3464S (2002).

71. Giovannucci, E. Diet, body weight, and colorectal cancer: a summary of the epidemiologic evidence. *J. Womens Health 2002* **12**, 173–182 (2003).

72. McGuckin, M. A., Eri, R., Simms, L. A., Florin, T. H. J. & Radford-Smith, G. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm. Bowel Dis.* **15**, 100–113 (2009).

73. Lin, S., Li, Y., Zamyatnin, A. A., Werner, J. & Bazhin, A. V. Reactive oxygen species and colorectal cancer. *J. Cell. Physiol.* **233**, 5119–5132 (2018).

74. Perše, M. Oxidative Stress in the Pathogenesis of Colorectal Cancer: Cause or Consequence? *BioMed Research International* (2013). doi:10.1155/2013/725710

75. Ziech, D., Franco, R., Pappa, A. & Panayiotidis, M. I. Reactive oxygen species (ROS)--induced genetic and epigenetic alterations in human carcinogenesis. *Mutat. Res.* **711**, 167–173 (2011).

76. Bartsch, H. & Nair, J. Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch. Surg.* **391**, 499–510 (2006).

77. Acharya, A., Das, I., Chandhok, D. & Saha, T. Redox regulation in cancer. *Oxid. Med. Cell. Longev.* **3**, 23–34 (2010).

78. Ko, C.-H., Shen, S.-C., Yang, L.-Y., Lin, C.-W. & Chen, Y.-C. Gossypol reduction of tumor growth through ROS-dependent mitochondria pathway in human colorectal carcinoma cells. *Int. J. Cancer* **121**, 1670–1679 (2007).

79. Girgin, F. *et al.* Effects of trimetazidine on oxidant/antioxidant status in trinitrobenzenesulfonic acid-induced chronic colitis. *J. Toxicol. Environ. Health A* **59**, 641–652 (2000).

80. Aiken, C. T., Kaake, R. M., Wang, X. & Huang, L. Oxidative stress-mediated regulation of proteasome complexes. *Mol. Cell. Proteomics MCP* **10**, R110.006924 (2011).

81. Tanida, S. *et al.* Involvement of oxidative stress and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in inflammatory bowel disease. *J. Clin. Biochem. Nutr.* **48**, 112–116 (2011).

82. Zhang, Q. *et al.* A superoxide dismutase/catalase mimetic nanomedicine for targeted therapy of inflammatory bowel disease. *Biomaterials* **105**, 206–221 (2016).

83. Crespo-Sanjuán, J. *et al.* Early detection of high oxidative activity in patients with adenomatous intestinal polyps and colorectal adenocarcinoma: myeloperoxidase and oxidized low-density lipoprotein in serum as new markers of oxidative stress in colorectal cancer. *Lab. Med.* **46**, 123–135 (2015).

84. Liu, H. *et al.* Redox Imbalance in the Development of Colorectal Cancer. *J. Cancer* **8**, 1586–1597 (2017).

85. Yang, Y., Karakhanova, S., Werner, J. & Bazhin, A. V. Reactive oxygen species in cancer biology and anticancer therapy. *Curr. Med. Chem.* **20**, 3677–3692 (2013).

86. Kirkali, G. *et al.* Abstract 1496: Oxidatively induced DNA base damage in colorectal carcinoma. *Cancer Res.* **70**, 1496–1496 (2010).

87. Lee, J.-H., Hwang, I., Kang, Y.-N., Choi, I.-J. & Kim, D.-K. Genetic Characteristics of Mitochondrial DNA Was Associated with Colorectal Carcinogenesis and Its Prognosis. *PLOS ONE* **10**, e0118612 (2015).

88. Yang, H.-Y. *et al.* Comparative proteomic analysis of cysteine oxidation in colorectal cancer patients. *Mol. Cells* **35**, 533–542 (2013).

89. Wei, X. *et al.* Activation of the JAK-STAT3 pathway is associated with the growth of colorectal carcinoma cells. *Oncol. Rep.* **31**, 335–341 (2014).

90. Sebio, A., Kahn, M. & Lenz, H.-J. The potential of targeting Wnt/β-catenin in colon cancer. *Expert Opin. Ther. Targets* **18**, 611–615 (2014).

91. Pandurangan, A. K. Potential targets for prevention of colorectal cancer: a focus on PI3K/Akt/mTOR and Wnt pathways. *Asian Pac. J. Cancer Prev. APJCP* **14**, 2201–2205 (2013).

92. Gupta, A., Rosenberger, S. F. & Bowden, G. T. Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines. *Carcinogenesis* **20**, 2063–2073 (1999).

93. Pelicci, P. G., Dalton, P. & Giorgio, M. The Other Face of ROS: a Driver of Stem Cell Expansion in Colorectal Cancer. *Cell Stem Cell* **12**, 635–636 (2013).

94. Schieber, M. & Chandel, N. S. ROS function in redox signaling and oxidative stress. *Curr. Biol. CB* **24**, R453-462 (2014).

95. Jabs, T. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol.* **57**, 231–245 (1999).

96. Eruslanov, E. & Kusmartsev, S. Identification of ROS Using Oxidized DCFDA and Flow-Cytometry. in *Advanced Protocols in Oxidative Stress II* (ed. Armstrong, D.) **594**, 57–72 (Humana Press, 2010).

97. Reuter, S., Gupta, S. C., Chaturvedi, M. M. & Aggarwal, B. B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **49**, 1603–1616 (2010).

98. Poyton, R. O., Ball, K. A. & Castello, P. R. Mitochondrial generation of free radicals and hypoxic signaling. *Trends Endocrinol. Metab. TEM* **20**, 332–340 (2009).

99. Liou, G.-Y. & Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **44**, 479–496 (2010).

100. Dikalov, S. I. & Harrison, D. G. Methods for Detection of Mitochondrial and Cellular Reactive Oxygen Species. *Antioxid. Redox Signal.* **20**, 372–382 (2014).

101. Gorrini, C., Harris, I. S. & Mak, T. W. Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov.* **12**, 931–947 (2013).

102. Storz, P. Reactive oxygen species in tumor progression. *Front. Biosci. J. Virtual Libr.* **10**, 1881–1896 (2005).

103. Szatrowski, T. P. & Nathan, C. F. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **51**, 794–798 (1991).

104. Sullivan, L. B. & Chandel, N. S. Mitochondrial reactive oxygen species and

cancer. *Cancer Metab.* **2**, 17 (2014).

105. Robertis, M. D. *et al.* The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J. Carcinog.* **10**, (2011).

106. Newmark, H. L. *et al.* Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. *Carcinogenesis* **30**, 88–92 (2009).

107. Richter, F., Newmark, H. L., Richter, A., Leung, D. & Lipkin, M. Inhibition of Western-diet induced hyperproliferation and hyperplasia in mouse colon by two sources of calcium. *Carcinogenesis* **16**, 2685–2689 (1995).

108. Johnson, R. L. & Fleet, J. C. Animal Models of Colorectal Cancer. *Cancer Metastasis Rev.* **32**, 39–61 (2013).

109. van Zeeland, A. A. Molecular dosimetry of chemical mutagens. Relationship between DNA adduct formation and genetic changes analyzed at the molecular level. *Mutat. Res. Mol. Mech. Mutagen.* **353**, 123–150 (1996).

110. Bissahoyo, A. *et al.* Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet. *Toxicol. Sci. Off. J. Soc. Toxicol.* **88**, 340–345 (2005).

111. Neufert, C., Becker, C. & Neurath, M. F. An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. *Nat. Protoc.* **2**, 1998–2004 (2007).

112. Fiala, E. S., Joseph, C., Sohn, O. S., el-Bayoumy, K. & Reddy, B. S. Mechanism of benzylselenocyanate inhibition of azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Res.* **51**, 2826–2830 (1991).

113. Rosenberg, D. W., Giardina, C. & Tanaka, T. Mouse models for the study of colon carcinogenesis. *Carcinogenesis* **30**, 183–196 (2009).

114. Fiala, E. S. *et al.* Non-alcohol dehydrogenase-mediated metabolism of methylazoxymethanol in the deer mouse, Peromyscus maniculatus. *Cancer Res.* **44**, 2885–2891 (1984).

115. Fiala, E. S., Kulakis, C., Christiansen, G. & Weisburger, J. H. Inhibition of the Metabolism of the Colon Carcinogen, Azoxymethane, by Pyrazole. *Cancer Res.* **38**, 4515–4521 (1978).

116. Nagasawa, H. T., Shirota, F. N. & Matsumoto, H. Decomposition of Methylazoxymethanol, the Aglycone of Cycasin, in D2O. *Nature* **236**, 234–235 (1972).

117. Feinberg, A. & Zedeck, M. S. Production of a Highly Reactive Alkylating Agent from the Organospecific Carcinogen Methylazoxymethanol by Alcohol Dehydrogenase. *Cancer Res.* **40**, 4446–4450 (1980).

118. Yamada, Y. *et al.* Frequent β-Catenin Gene Mutations and Accumulations of the Protein in the Putative Preneoplastic Lesions Lacking Macroscopic Aberrant Crypt Foci Appearance, in Rat Colon Carcinogenesis. *Cancer Res.* **60**, 3323–3327 (2000).

119. Su, L. K. *et al.* Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* **256**, 668–670 (1992).

120. Luongo, C., Moser, A. R., Gledhill, S. & Dove, W. F. Loss of Apc+ in intestinal adenomas from Min mice. *Cancer Res.* **54**, 5947–5952 (1994).

121. Yamada, Y. & Mori, H. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci.* **98**, 6–10 (2007).

122. Irving, A. A. *et al.* The utility of Apc-mutant rats in modeling human colon cancer. *Dis. Model. Mech.* **7**, 1215–1225 (2014).

123. Klintrup, K. *et al.* Inflammation and prognosis in colorectal cancer. *Eur. J. Cancer Oxf. Engl. 1990* **41**, 2645–2654 (2005).

124. Leitch, E. F. *et al.* Comparison of the prognostic value of selected markers of the systemic inflammatory response in patients with colorectal cancer. *Br. J. Cancer* **97**, 1266–1270 (2007).

125. Grizzi, F., Bianchi, P., Malesci, A. & Laghi, L. Prognostic value of innate and adaptive immunity in colorectal cancer. *World J. Gastroenterol. WJG* **19**, 174–184 (2013).

126. Warrington, R., Watson, W., Kim, H. L. & Antonetti, F. R. An introduction to immunology and immunopathology. *Allergy Asthma Clin. Immunol. Off. J. Can. Soc.*

Allergy Clin. Immunol. **7**, S1 (2011).

127. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819–826 (2007).

128. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* **125**, S33–S40 (2010).

129. Melvold, R. W. & Sticca, R. P. Basic and tumor immunology: a review. *Surg. Oncol. Clin. N. Am.* **16**, 711–735, vii (2007).

130. Pagès, F. *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N. Engl. J. Med.* **353**, 2654–2666 (2005).

131. Zhong, J., Xu, J.-F., Yang, P., Liang, Y. & Wang, C.-Y. Innate Immunity in the Recognition of β-Cell Antigens in Type 1 Diabetes. in *Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy* (ed. Wagner, D.) (InTech, 2011).

132. Tatsumi, T. *et al.* Expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on human hepatocellular carcinoma. *Hepatology* **25**, 1108–1114 (1997).

133. Koido, S. Immunotherapy for colorectal cancer. *World J. Gastroenterol.* **19**, 8531 (2013).

134. Shahinian, A. *et al.* Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609–612 (1993).

135. Borowski, A. B. *et al.* Memory CD8+ T cells require CD28 costimulation. *J. Immunol. Baltim. Md 1950* **179**, 6494–6503 (2007).

136. Lim, W., Gee, K., Mishra, S. & Kumar, A. Regulation of B7.1 Costimulatory Molecule Is Mediated by the IFN Regulatory Factor-7 through the Activation of JNK in Lipopolysaccharide-Stimulated Human Monocytic Cells. *J. Immunol.* **175**, 5690–5700 (2005).

137. Gauna, A. E. *et al.* Dysregulated co-stimulatory molecule expression in a Sjögren's syndrome mouse model with potential implications by microRNA-146a. *Mol. Immunol.* (2015). doi:10.1016/j.molimm.2015.09.027

138. Collins, A. V. *et al.* The interaction properties of costimulatory molecules

revisited. *Immunity* **17**, 201–210 (2002).

139. Vasaturo, A. *et al.* Clinical implications of co-inhibitory molecule expression in the tumor microenvironment for DC vaccination: a game of stop and go. *Tumor Immun.* **4**, 417 (2013).

140. Tirapu, I. *et al.* Low Surface Expression of B7-1 (CD80) Is an Immunoescape Mechanism of Colon Carcinoma. *Cancer Res.* **66**, 2442–2450 (2006).

141. Burnet, F. M. The concept of immunological surveillance. *Prog. Exp. Tumor Res.* **13**, 1–27 (1970).

142. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **331**, 1565–1570 (2011).

143. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* **3**, 991–998 (2002).

144. Monjazeb, A. M. *et al.* Immunoediting and Antigen Loss: Overcoming the Achilles Heel of Immunotherapy with Antigen Non-Specific Therapies. *Front. Oncol.* **3**, (2013).

145. Koebel, C. M. *et al.* Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* **450**, 903–907 (2007).

146. Driessens, G., Kline, J. & Gajewski, T. F. Costimulatory and coinhibitory receptors in anti-tumor immunity. *Immunol. Rev.* **229**, 126–144 (2009).

147. Chen, D. S. & Mellman, I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* **39**, 1–10 (2013).

148. Motz, G. T. & Coukos, G. Deciphering and reversing tumor immune suppression. *Immunity* **39**, 61–73 (2013).

149. Wang, L. *et al.* Immune evasion of mantle cell lymphoma: expression of B7-H1 leads to inhibited T-cell response to and killing of tumor cells. *Haematologica* **98**, 1458–1466 (2013).

150. Predina, J. *et al.* Changes in the local tumor microenvironment in recurrent cancers may explain the failure of vaccines after surgery. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E415-424 (2013).

151. Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480**, 480–489 (2011).

152. Ravoori, S. & PhD. Can We Treat Colorectal Cancer With Immunotherapy? *CANCER RESEARCH Catalyst* (2016). Available at: http://blog.aacr.org/can-treat-colorectal-cancer-immunotherapy/. (Accessed: 1st December 2016)

153. Jacobs, J., Smits, E., Lardon, F., Pauwels, P. & Deschoolmeester, V. Immune Checkpoint Modulation in Colorectal Cancer: What’s New and What to Expect. *J. Immunol. Res.* **2015**, e158038 (2015).

154. Vilar, E. & Gruber, S. B. Microsatellite instability in colorectal cancer—the stable evidence. *Nat. Rev. Clin. Oncol.* **7**, 153–162 (2010).

155. FDA Approves Immunotherapy Combination for Metastatic Colorectal Cancer. *Cure Today* Available at: https://www.curetoday.com/articles/fda-approves-immunotherapy-combination-for-metastatic-colorectal-cancer. (Accessed: 24th November 2018)

156. Bacac, M. *et al.* A Novel Carcinoembryonic Antigen T-Cell Bispecific Antibody (CEA TCB) for the Treatment of Solid Tumors. *Clin. Cancer Res.* **22**, 3286–3297 (2016).

157. Bacac, M., Klein, C. & Umana, P. CEA TCB: A novel head-to-tail 2:1 T cell bispecific antibody for treatment of CEA-positive solid tumors. *Oncoimmunology* **5**, (2016).

158. Nakazawa, A. *et al.* Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucosa. *Gastroenterology* **117**, 536–545 (1999).

159. Fleischer, J. *et al.* Differential expression and function of CD80 (B7-1) and CD86 (B7-2) on human peripheral blood monocytes. *Immunology* **89**, 592–598 (1996).

160. McLellan, A. D., Starling, G. C., Williams, L. A., Hock, B. D. & Hart, D. N. J.

Activation of human peripheral blood dendritic cells induces the CD86 co-stimulatory molecule. *Eur. J. Immunol.* **25**, 2064–2068 (1995).

161. Mancino, A. *et al.* Divergent effects of hypoxia on dendritic cell functions. *Blood* **112**, 3723–3734 (2008).

162. Lahat, N. *et al.* Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-α secretion. *J. Leukoc. Biol.* **74**, 197–205 (2003).

163. Donepudi, M., Raychaudhuri, P., Bluestone, J. A. & Mokyr, M. B. Mechanism of Melphalan-Induced B7-1 Gene Expression in P815 Tumor Cells. *J. Immunol.* **166**, 6491–6499 (2001).

164. Kanada, S. *et al.* Critical role of transcription factor PU.1 in the expression of CD80 and CD86 on dendritic cells. *Blood* **117**, 2211–2222 (2011).

165. Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* **12**, 252–264 (2012).

166. Galamb, O. *et al.* Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor. *Br. J. Cancer* **102**, 765–773 (2010).

167. Fassan, M. *et al.* Assessment of intratumor immune-microenvironment in colorectal cancers with extranodal extension of nodal metastases. *Cancer Cell Int.* **18**, (2018).

168. Irrazábal, T., Belcheva, A., Girardin, S. E., Martin, A. & Philpott, D. J. The Multifaceted Role of the Intestinal Microbiota in Colon Cancer. *Mol. Cell* **54**, 309–320 (2014).

169. Satokari, R., Grönroos, T., Laitinen, K., Salminen, S. & Isolauri, E. Bifidobacterium and Lactobacillus DNA in the human placenta. *Lett. Appl. Microbiol.* **48**, 8–12 (2009).

170. Jiménez, E. *et al.* Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr. Microbiol.* **51**, 270–274 (2005).

171. Faith, J. J. *et al.* The long-term stability of the human gut microbiota. *Science* **341**, 1237439 (2013).

172. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).

173. Qin, J. *et al.* A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59–65 (2010).

174. Costea, P. I. *et al.* Enterotypes in the landscape of gut microbial community composition. *Nat. Microbiol.* **3**, 8–16 (2018).

175. Robles Alonso, V. & Guarner, F. Linking the gut microbiota to human health. *Br. J. Nutr.* **109 Suppl 2**, S21-26 (2013).

176. Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. Host-bacterial mutualism in the human intestine. *Science* **307**, 1915–1920 (2005).

177. Mazmanian, S. K., Liu, C. H., Tzianabos, A. O. & Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122**, 107–118 (2005).

178. Tlaskalová-Hogenová, H. *et al.* The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cell. Mol. Immunol.* **8**, 110–120 (2011).

179. Reddy, B. S., Weisburger, J. H., Narisawa, T. & Wynder, E. L. Colon carcinogenesis in germ-free rats with 1,2-dimethylhydrazine and N-methyl-n'-nitro-N-nitrosoguanidine. *Cancer Res.* **34**, 2368–2372 (1974).

180. Reddy, B. S. *et al.* Colon carcinogenesis with azoxymethane and dimethylhydrazine in germ-free rats. *Cancer Res.* **35**, 287–290 (1975).

181. Vannucci, L. *et al.* Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. *Int. J. Oncol.* **32**, 609–617 (2008).

182. Dove, W. F. *et al.* Intestinal neoplasia in the ApcMin mouse:

independence from the microbial and natural killer (beige locus) status. *Cancer Res.* **57**, 812–814 (1997).

183. Chen, G. Y., Shaw, M. H., Redondo, G. & Núñez, G. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. *Cancer Res.* **68**, 10060–10067 (2008).

184. Grivennikov, S. I. *et al.* Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491**, 254–258 (2012).

185. Klimesova, K. *et al.* Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M-deficient mice. *Inflamm. Bowel Dis.* **19**, 1266–1277 (2013).

186. Sommer, F. & Bäckhed, F. The gut microbiota--masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238 (2013).

187. Duvallet, C., Gibbons, S. M., Gurry, T., Irizarry, R. A. & Alm, E. J. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nat. Commun.* **8**, 1784 (2017).

188. Hibberd, A. A. *et al.* Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. *BMJ Open Gastroenterol.* **4**, (2017).

189. Gagnière, J. *et al.* Gut microbiota imbalance and colorectal cancer. *World J. Gastroenterol.* **22**, 501 (2016).

190. Chen, H.-M. *et al.* Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma. *Am. J. Clin. Nutr.* **97**, 1044–1052 (2013).

191. Kostic, A. D. *et al.* Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res.* **22**, 292–298 (2012).

192. Geng, J., Fan, H., Tang, X., Zhai, H. & Zhang, Z. Diversified pattern of the human colorectal cancer microbiome. *Gut Pathog.* **5**, 2 (2013).

193. Kamada, N., Seo, S.-U., Chen, G. Y. & Núñez, G. Role of the gut microbiota in immunity and inflammatory disease. *Nat. Rev. Immunol.* **13**, 321–335 (2013).

194. Abreu, M. T., Fukata, M. & Arditi, M. TLR signaling in the gut in health and disease. *J. Immunol. Baltim. Md 1950* **174**, 4453–4460 (2005).

195. Ranjan, P. *et al.* Cytoplasmic nucleic acid sensors in antiviral immunity. *Trends Mol. Med.* **15**, 359–368 (2009).

196. Stier, S. *et al.* Combinations of TLR Ligands: A Promising Approach in Cancer Immunotherapy, Combinations of TLR Ligands: A Promising Approach in Cancer Immunotherapy. *J. Immunol. Res. J. Immunol. Res.* **2013**, **2013**, e271246 (2013).

197. Yesudhas, D., Gosu, V., Anwar, M. A. & Choi, S. Multiple Roles of Toll-Like Receptor 4 in Colorectal Cancer. *Front. Immunol.* **5**, (2014).

198. Rakoff-Nahoum, S. & Medzhitov, R. Toll-like receptors and cancer. *Nat. Rev. Cancer* **9**, 57–63 (2009).

199. O'Neill, L. A., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors [mdash] redefining innate immunity. *Nat. Rev. Immunol.* **13**, 453–460 (2013).

200. Pradere, J.-P., Dapito, D. H. & Schwabe, R. F. The Yin and Yang of Toll-like receptors in cancer. *Oncogene* **33**, 3485–3495 (2014).

201. Li, T.-T., Ogino, S. & Qian, Z. R. Toll-like receptor signaling in colorectal cancer: Carcinogenesis to cancer therapy. *World J. Gastroenterol. WJG* **20**, 17699–17708 (2014).

202. Wang, D. *et al.* Antitumor Activity and Immune Response Induction of a Dual Agonist of Toll-Like Receptors 7 and 8. *Mol. Cancer Ther.* **9**, 1788–1797 (2010).

203. Fukata, M. *et al.* Toll-Like Receptor-4 Promotes the Development of Colitis-Associated Colorectal Tumors. *Gastroenterology* **133**, 1869-1869.e14 (2007).

204. Li, Y. *et al.* Constitutive TLR4 signalling in intestinal epithelium reduces tumor load by increasing apoptosis in $APC^{Min/+}$ mice. *Oncogene* **33**, 369–377 (2014).

205. Grossmann, J. *et al.* Progress on isolation and short-term ex-vivo culture of highly purified non-apoptotic human intestinal epithelial cells (IEC). *Eur. J. Cell Biol.* **82**, 262–270 (2003).

206. Al-Numair, K. S. *et al.* Dietary folate protects against azoxymethane-

induced aberrant crypt foci development and oxidative stress in rat colon. *Exp. Biol. Med.* **236**, 1005–1011 (2011).

207. Waly, M. I. *et al.* Pomegranate (Punica granatum) Peel Extract Efficacy as a Dietary Antioxidant against Azoxymethane-Induced Colon Cancer in Rat. *Asian Pac. J. Cancer Prev.* **13**, 4051–4055 (2012).

208. Waly, M. I. *et al.* Amelioration of azoxymethane induced-carcinogenesis by reducing oxidative stress in rat colon by natural extracts. *BMC Complement. Altern. Med.* **14**, 60 (2014).

209. Geiger, B., Cohen-Noyman, E., Bar, J. & Oren, M. Attenuation of the p53 response to DNA damage by high cell density. *Oncogene* **23**, 2128 (2004).

210. Awasthi, P., Foiani, M. & Kumar, A. ATM and ATR signaling at a glance. *J. Cell Sci.* **128**, 4255–4262 (2015).

211. Borodkina, A., Shatrova, A., Abushik, P., Nikolsky, N. & Burova, E. Interaction between ROS dependent DNA damage, mitochondria and p38 MAPK underlies senescence of human adult stem cells. *Aging* **6**, 481–495 (2014).

212. Li, H. *et al.* Pharmacological activation of p53 triggers anticancer innate immune response through induction of ULBP2. *Cell Cycle* **10**, 3346–3358 (2011).

213. Oleinik, N. V., Krupenko, N. I. & Krupenko, S. A. Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. *Oncogene* **26**, 7222 (2007).

214. Ashley, A. K. & Kemp, C. J. DNA-PK, ATM, and ATR: PIKKing on p53. *Cell Cycle Georget. Tex* **17**, 275–276 (2018).

215. Souza, M. A. *et al.* Antioxidant activity elicited by low dose of caffeine attenuates pentylenetetrazol-induced seizures and oxidative damage in rats. *Neurochem. Int.* **62**, 821–830 (2013).

216. Tellone, E. *et al.* Chapter 92 - Antioxidant Activity of Caffeine: A Focus on Human Red Blood Cells and Correlations with Several Neurodegenerative Disorders. in *Coffee in Health and Disease Prevention* (ed. Preedy, V. R.) 835–842 (Academic Press, 2015). doi:10.1016/B978-0-12-409517-5.00092-9

217. Son, Y., Kim, S., Chung, H.-T. & Pae, H.-O. Reactive Oxygen Species in the Activation of MAP Kinases. in *Methods in Enzymology* **528**, 27–48 (Elsevier, 2013).

218. Son, Y. *et al.* Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? *Journal of Signal Transduction* (2011). doi:10.1155/2011/792639

219. Nakagawa, H. & Maeda, S. Molecular Mechanisms of Liver Injury and Hepatocarcinogenesis: Focusing on the Role of Stress-Activated MAPK. *Pathol. Res. Int.* **2012**, e172894 (2012).

220. MENG, A., ZHANG, X. & SHI, Y. Role of p38 MAPK and STAT3 in lipopolysaccharide-stimulated mouse alveolar macrophages. *Exp. Ther. Med.* **8**, 1772–1776 (2014).

221. Riebe, C., Pries, R., Schroeder, K. N. & Wollenberg, B. Phosphorylation of STAT3 in Head and Neck Cancer Requires p38 MAPKinase, whereas Phosphorylation of STAT1 Occurs via a Different Signaling Pathway. *Anticancer Res.* **31**, 3819–3825 (2011).

222. Zauberman, A., Zipori, D., Krupsky, M. & Ben-Levy, R. Stress activated protein kinase p38 is involved in IL-6 induced transcriptional activation of STAT3. *Oncogene* **18**, 3886–3893 (1999).

223. Deplancke, B. & Gaskins, H. R. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* **73**, 1131S-1141S (2001).

224. Ubeda, C. *et al.* Vancomycin-resistant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J. Clin. Invest.* **120**, 4332–4341 (2010).

225. Corrêa-Oliveira, R., Fachi, J. L., Vieira, A., Sato, F. T. & Vinolo, M. A. R. Regulation of immune cell function by short-chain fatty acids. *Clin. Transl. Immunol.* **5**, e73 (2016).

226. Chen, L. *et al.* Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* **71**, 1093–1102 (1992).

227. Townsend, S. E. & Allison, J. P. Tumor rejection after direct costimulation

of CD8+ T cells by B7-transfected melanoma cells. *Science* **259**, 368–370 (1993).

228. Ramarathinam, L., Castle, M., Wu, Y. & Liu, Y. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J. Exp. Med.* **179**, 1205–1214 (1994).

229. Wu, T. C., Huang, A. Y., Jaffee, E. M., Levitsky, H. I. & Pardoll, D. M. A reassessment of the role of B7-1 expression in tumor rejection. *J. Exp. Med.* **182**, 1415–1421 (1995).

230. Morel, A. *et al.* Gamma-ray irradiation induces B7.1 costimulatory molecule neoexpression in various murine tumor cells. *Cancer Immunol. Immunother. CII* **46**, 277–282 (1998).

231. Wakem, P. *et al.* Allergens and irritants transcriptionally upregulate CD80 gene expression in human keratinocytes. *J. Invest. Dermatol.* **114**, 1085–1092 (2000).

232. Satoh, S. *et al.* Glomerular endothelium exhibits enhanced expression of costimulatory adhesion molecules, CD80 and CD86, by warm ischemia/reperfusion injury in rats. *Lab. Investig. J. Tech. Methods Pathol.* **82**, 1209–1217 (2002).

233. Avalle, L., Pensa, S., Regis, G., Novelli, F. & Poli, V. STAT1 and STAT3 in tumorigenesis. *JAK-STAT* **1**, 65–72 (2012).

234. Grivennikov, S. *et al.* IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer. *Cancer Cell* **15**, 103–113 (2009).

235. Musteanu, M. *et al.* Stat3 is a negative regulator of intestinal tumor progression in Apc(Min) mice. *Gastroenterology* **138**, 1003-1011.e1–5 (2010).

236. Ziegler, P. K. *et al.* Mitophagy in Intestinal Epithelial Cells Triggers Adaptive Immunity during Tumorigenesis. *Cell* **174**, 88-101.e16 (2018).

237. Center, M. M., Jemal, A. & Ward, E. International trends in colorectal cancer incidence rates. *Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol.* **18**, 1688–1694 (2009).

238. Center, M. M., Jemal, A., Smith, R. A. & Ward, E. Worldwide variations in colorectal cancer. *CA. Cancer J. Clin.* **59**, 366–378 (2009).

239. Nistal, E., Fernández-Fernández, N., Vivas, S. & Olcoz, J. L. Factors Determining Colorectal Cancer: The Role of the Intestinal Microbiota. *Front. Oncol.* **5**, (2015).

240. O'Leary, D. P. *et al.* TLR-4 Signalling Accelerates Colon Cancer Cell Adhesion via NF-κB Mediated Transcriptional Up-Regulation of Nox-1. *PLoS ONE* **7**, e44176 (2012).

241. Luddy, K. A., Robertson-Tessi, M., Tafreshi, N. K., Soliman, H. & Morse, D. L. The Role of Toll-Like Receptors in Colorectal Cancer Progression: Evidence for Epithelial to Leucocytic Transition. *Front. Immunol.* **5**, (2014).

242. Luo, J.-L., Maeda, S., Hsu, L.-C., Yagita, H. & Karin, M. Inhibition of NF-κB in cancer cells converts inflammation- induced tumor growth mediated by TNFα to TRAIL-mediated tumor regression. *Cancer Cell* **6**, 297–305 (2004).

243. Pidgeon, G. P. *et al.* The role of endotoxin/lipopolysaccharide in surgically induced tumour growth in a murine model of metastatic disease. *Br. J. Cancer* **81**, 1311–1317 (1999).

244. Harmey, J. H. *et al.* Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. *Int. J. Cancer* **101**, 415–422 (2002).

245. Andreani, V., Gatti, G., Simonella, L., Rivero, V. & Maccioni, M. Activation of Toll-like receptor 4 on tumor cells in vitro inhibits subsequent tumor growth in vivo. *Cancer Res.* **67**, 10519–10527 (2007).

246. Otto, F. *et al.* Phase II trial of intravenous endotoxin in patients with colorectal and non-small cell lung cancer. *Eur. J. Cancer* **32**, 1712–1718 (1996).

247. Castellarin, M. *et al.* Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res.* **22**, 299–306 (2012).

248. Marchesi, J. R. *et al.* Towards the Human Colorectal Cancer Microbiome. *PLOS ONE* **6**, e20447 (2011).

249. Fang, H. *et al.* TLR4 is essential for dendritic cell activation and anti-tumor T-cell response enhancement by DAMPs released from chemically stressed cancer cells. *Cell. Mol. Immunol.* **11**, 150–159 (2014).

250. Lu, Y.-C., Yeh, W.-C. & Ohashi, P. S. LPS/TLR4 signal transduction pathway. *Cytokine* **42**, 145–151 (2008).