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DOTTORATO DI RICERCA IN INFIAMMAZIONE, IMMUNITA' E CANCRO
XXXI CICLO

INFLUENCE OF MICRO- AND MACROENVIRONMENT IN COLON CANCER

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SOMMARIO

Un numero sempre maggiore di lavori evidenzia il contributo del sistema immunitario nello sviluppo e nella progressione del cancro. Infatti, dati recenti hanno dimostrato che ogni tumore è caratterizzato dalla presenza di un infiltrato di cellule infiammatorie che costituisce parte del microambiente tumorale. Inoltre, la progressione tumorale coinvolge ed è influenzata da organi situati in *loci* lontani dal sito del tumore primario. Tale coinvolgimento è dovuto al rilascio di fattori solubili, quali citochine, ormoni e molecole "decoy". E' quindi di fondamentale importanza considerare non solo il ruolo del microambiente tumorale, ma anche quello del macroambiente.

Recentemente, sono stati studiati i cambiamenti del macroambiente tumorale che caratterizzano il CRC nel modello genetico murino $Apc^{Min/+}$, nel quale è stato riscontrato un aumento statisticamente significativo dei livelli di IgA rispetto ai topi WT di controllo. A partire da questi dati, in questo lavoro di tesi sono stati studiati i possibili meccanismi responsabili dell'aumento di IgA nel contesto del CRC. I dati ottenuti hanno dimostrato che la maggiore tendenza a produrre IgA non è un tratto generale che caratterizza tutti i modelli murini di cancro al colon retto analizzati, ma evidenziano che la produzione aumentata di IgA è peculiare del modello murino $Apc^{Min/+}$. Inoltre, la presenza di elevati livelli di IgA in contesto tumorale rispetto a quelli rilevati in condizioni fisiologiche, è stata esclusa in diversi modelli murini di cancro non associati al tratto gastro-intestinale.

Molte malattie intestinali sono state associate alla presenza di un'aumentata permeabilità intestinale. In questo lavoro di tesi, viene proposto un meccanismo secondo il quale i danni indotti dal tumore a livello della barriera intestinale, determinano una aumentata traslocazione batterica che può, di conseguenza, essere associata alla progressione tumorale.

Nella fattispecie, nella presente tesi è stata dimostrata la presenza di un più alto livello di batteri nel fegato di topi $Apc^{Min/+}$, rispetto a quello riscontrato in topi WT. Nello specifico, nei topi $Apc^{Min/+}$ è stata rilevata la presenza di *Bacteroides*

fragilis, che è invece assente nei topi di controllo WT. Recenti dati presenti in letteratura attribuiscono un ruolo tumorigenico a *B. fragilis*; la presenza dello stesso nel microbioma di topi $Apc^{Min/+}$ è indicativa quindi di un potenziale cancerogeno. I dati presentati suggeriscono quindi un'associazione diretta o indiretta tra il microbioma intestinale e il contesto tumorale di topi $Apc^{Min/+}$ rispetto a quello di topi sani.

Lo studio del microambiente immunitario ha principalmente focalizzato l'attenzione sul ruolo delle cellule T immunosoppressive che inibiscono la risposta immunitaria anti-tumorale. Al contrario, il ruolo delle cellule B non è ancora chiaro e richiede pertanto ulteriori studi.

In questo lavoro di tesi, è stata rilevata la presenza di un abbondante accumulo di cellule MDSC nel microambiente tumorale del CRC e l'abilità delle MDSC purificate dalla milza di topi con tumore MC38 di influenzare i linfociti B, inducendo un fenotipo immunosoppressivo. In particolare, è stata rivelata l'abilità delle MDSC di indurre l'espansione di cellule B competenti alla produzione di IL-10, nonchè l'espressione dei marker FasL, PD-L1 e IgA in cellule B naïve.

Questo lavoro di tesi potrebbe, pertanto, chiarire i meccanismi che caratterizzano il microambiente e il macroambiente tumorale del cancro al colon retto per lo sviluppo di nuove potenziali strategie immuno-terapeutiche.

ABSTRACT

An increasing body of evidence supports a contribute of the immune system in cancer development and progression, in fact recent data have demonstrated that, virtually, every tumor has an inflammatory cell infiltrate as part of a complex tumor microenvironment (TME). Cancer progression is also directed by the involvement of organs located at distant sites from the primary tumor due to the release of tumor-derived soluble factors (TDSFs). Therefore, it is very important to consider not only the role of the tumor-associated stroma, which is known as TME, but also the creation of a network even with distal compartments due to the release of many TDSFs leading to the generation of the so-called tumor macroenvironment.

Recently, the macroenvironment changes have been underlined in the genetic model of colon cancer $Apc^{Min/+}$, where a significantly higher total IgA levels in $Apc^{Min/+}$ tumor-bearing mice has been demonstrated. In this work, the possible mechanisms of the IgA skewing have been dissected in the colorectal cancer (CRC) context. The data demonstrated that the sole IgA skewing is not a tract present in all CRC models, but that it is peculiar of the $Apc^{Min/+}$ mice; the presence of the IgA sewing in mouse tumor models not associated with the intestinal tract was also excluded.

Considering the association of several intestinal diseases with increased intestinal permeability, the damaged intestinal barrier, which can lead to the translocation of bacteria, was proposed to be associated with the tumor progression. A higher presence of bacterial level in the $Apc^{Min/+}$ compared to the WT mice was detected, indicating an increased microbial translocation to the liver. Furthermore, the presence of *Bacteroides fragilis* in the $Apc^{Min/+}$ mice was found, a type of bacteria that was instead absent in the WT counterpart. Our data indicate the carcinogenic potential of the microbiota in the $Apc^{Min/+}$ tumor context, since it has been demonstrated a tumorigenic role for *B. fragilis*. The data, herein presented, suggest a direct or indirect association of the microbiota in the $Apc^{Min/+}$ tumor setting compared to the healthy mice.

The progresses in the study of the immune microenvironment have mainly highlighted the role of immunosuppressive T cell in the inhibition of the anti-tumor immune response. Instead, the role of B cells is less well understood. In the present work, the presence of a accumulation of MDSCs in the CRC TME and the ability of splenic MDSCs from MC38 tumor-bearing mice to affect the phenotype of B cells, inducing a shift towards an immunosuppressive B cell phenotype, have been highlighted. In particular, the ability of splenic MDSCs isolated from MC38 tumor-bearing mice was reported to induce the expansion of the IL-10 competent B cells and to increase the expression of FasL, PD-L1, and IgA on naïve B cells. This work might be, therefore, helpful for clarifying the alteration of the systemic tumor environment occurring in CRC and for the development of new potential immunotherapeutic strategies.

1. INTRODUCTION

The immune system is the body's defence comprising many structures, tissues, organs and many cells with different biological activity that act together to defend our body from harmful organisms, foreign substances and constitute a protection from diseases.

The immune system can be classified into distinct arms: the innate immunity and the adaptive immunity, the latter, depending on the components involved, may be subdivided in humoral immunity and cell-mediated immunity.

The innate immunity is the first line of defence and includes chemical molecules such as histamine, physical barriers such as skin, the gastrointestinal tract, the respiratory tract, the nasopharynx or the cilia; cells, such as macrophages and the natural killer cells (NK), blood proteins including those of the complement system, the numerous cytokines and chemokines, which regulate a multiplicity of activities of the innate immune cells. On the other hand, the main components of the adaptive immune response are the lymphocytes that can be divided in two different subpopulations depending on the antigen receptor that they have, B and T lymphocytes. The B lymphocytes, following the activation, can differentiate in plasmacells that can produce a soluble form of the their antitgen receptor that is called antibody. The T cells can be divided in two major subpopulation depending on the structure of their recepotors, αα or γδ and, usually, they recognize antigens presented by the major histocompatibility complex (MHC) or non-canonical MHC. An additional type of lymphocytes, not antigen specific, critical to the innate immune systems, is the NK cells. NK cells play a role that shows analogy with cytotoxic T cells; they deliver a rapid response to viral-infected cells, acting few days after infection.

Typically, T cells detect MHC presented on infected cell surfaces, triggering cytokine release, causing lysis or apoptosis. NK cells have a unique and opposite way of recognizing infected cells, in fact, they have the capability to distinguish stressed cells in the absence of MHC on their surface. Furthermore, the immune system is finely organized to prevent unwanted effector immune response, it

includes regulatory populations such as T (Treg) and B cells (Breg) with anti-inflammatory properties that turn off the immune response maintaining the homeostasis and preventing autoimmune responses.

1.1. Cancer and its microenvironment

1.1.1. The hallmarks of cancer

Cancer is a heterogeneous disease in which normal cells transform into malignant cells and aberrantly proliferate into the host. Despite every type of cancer possess distinctive properties, some molecular and phenotypic characteristics are shared by the different types of cancer and are grouped as hallmarks of cancer (Figure 1) (Hanahan and Weinberg, 2000).

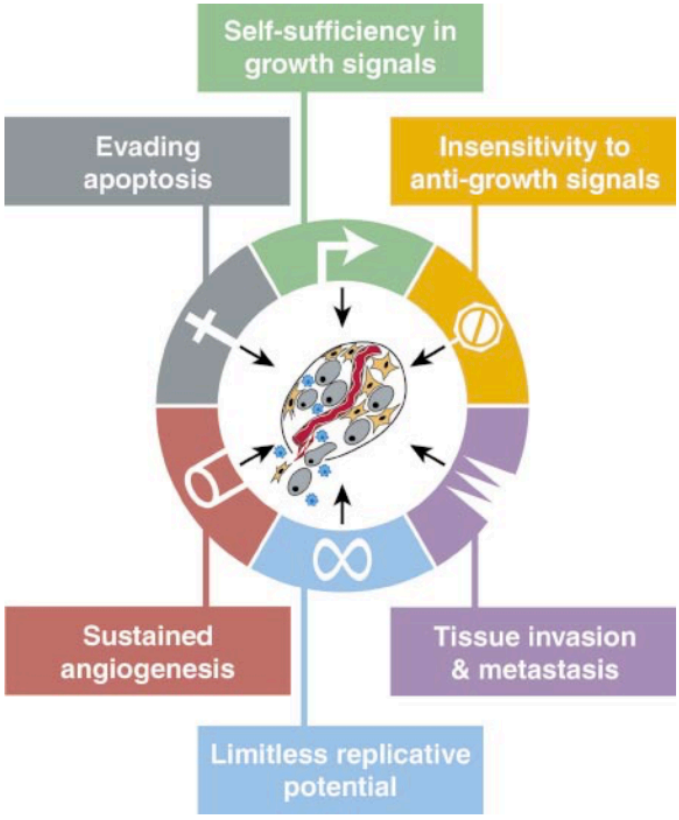


Figure 1. The hallmarks of cancer. Set of acquired functional capabilities of cancer cells during their development (Hanahan and Weinberg, 2000).

Cancer cells take advantage of several mechanisms to evade destruction by the immune system and to favour tumor development and progression. Cancer cells can support proliferative signaling determining continuous and rapid proliferation, to escape growth suppressors, to resist cell death and to enable replicative immortality. Therefore, through these mechanisms, cancer cells can sustain chronic proliferation and evade homeostatic controls.

Another hallmark of cancer is angiogenesis: the formation of new blood vessels in the tumor mass guarantees cells a path to enter the circulation and move everywhere in the host. This aptitude of cancer cells leads to another fundamental characteristic of tumor: the ability to invade the surrounding tissue and metastasize to distant organs (Nguyen et al., 2009).

Finally, cancer cells are able to elude the recognition by the host immune system promoting the establishment of an immunosuppressive microenvironment that hampers the proficient immune response against the tumor.

Among the aspects that characterize the tumor microenvironment, it is also important to consider the presence of cancer stem cells (CSCs), a chemotherapy-resistant subpopulation with the ability to sustain their turnover and to generate differentiate cells which form the bulk of the tumor (Lobo et al., 2007).

1.1.2. Tumor-infiltrating inflammatory cells and immunosuppression

The anti-tumor response is orchestrated by $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ Th_1 (T helper) cells, B cells and NK cells, which cover key roles in preventing tumor progression. Likewise, the infiltration of the immune cells in the tumor has been correlated with increased overall survival (OS) and decreased relapse after therapy (Pagès et al., 2010).

However, conversely, cancer cells are often able to evade immune surveillance in order to promote cancer progression. To explain this mechanism Schreiber and colleagues formulated the immunoediting hypothesis (Schreiber et al., 2011). It describes tumor development in three phases (figure 2):

1. the elimination phase consists in activating the immune response against cancer;
2. the equilibrium phase in which there is the establishment of a

balance between the eradication of cancer cells and the survival of mutated and more resistant clones of cancer cells which further expand;

3. the escape phase: tumor cells can become less immunogenic by expressing fewer aberrant molecules or down-regulating the antigen-presenting machinery. Through these mechanisms, cancer cells become fully competent in evading the host immune response, therefore, favoring the tumor progression.

Furthermore, TDSFs or cell-membrane associated molecules facilitate the escape from immune attack, allowing progression and metastasis (Balkwill et al., 2005).

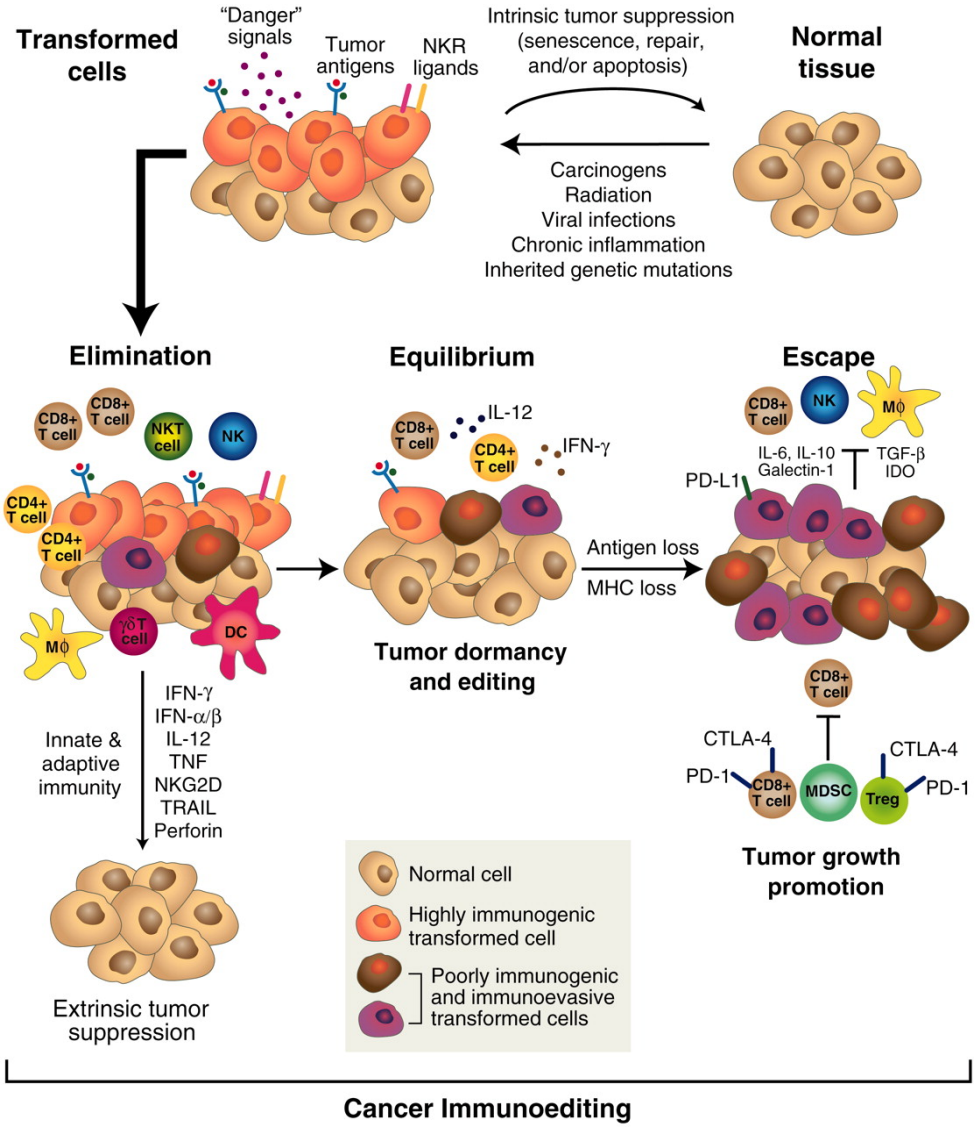


Figure 2. The cancer immunoediting hypothesis. The cancer immunoediting process is composed by three sequential phases: the elimination, the equilibrium and the escape phase. During the elimination phase, innate and adaptive immunity act to fight the tumor development. If the cancer cells are not completely eliminated during the first phase, they can enter the equilibrium phase, in which T cells, IL-12, and IFN-γ try to maintain tumor cells in a state of functional quiescence, blocking the tumor growth. New tumor cell variants appear: they are fully competent in evading the host immune response and are able to create an immunosuppressive microenvironment. Tumor cells can now enter the escape phase, in which they are no longer sensitive to the immune mechanisms and therefore favour the tumor progression (Schreiber et al., 2011).

In the last decade, a profuse literature has in fact highlighted the presence of tumor-infiltrating immune cells as critical determinants for suppressing the host immune response and for favouring instead the tumor progression and metastasis (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Schreiber et al., 2011). In light of these aspects, immunosuppressive subsets infiltrating the tumor correlated with poor prognosis and are also responsible for the reduced efficacy of anti-cancer therapies (Mitchem et al., 2013; Zou, 2005).

1.1.3. Immune checkpoints

The immune system is finely organized to guarantee immune homeostasis through the presence of modulatory molecules called "immune checkpoints" which modulate the immune response by preserving the equilibrium between co-stimulatory and inhibitory signals. This balance can be altered by uncontrolled immune response to pathogens or due to the presence of mutated self-antigens, which cause tissue damage and autoimmune diseases.

The main responsibile of immune effector functions are activated T cells, therefore they express several co-inhibitory receptors such as CTLA-4, PD-1, LAG-3, TIM-3, 4-1BB.

CTLA-4

Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), also known as CD152, is a transmembrane receptor expressed on recently activated T cells. It is homologous to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively expressed on recently activated T cells. The CTLA-4 binding on the B7-1 and B7-2 molecules (respectively CD80 and CD86) expressed on antigen presenting cells (APCs) sends an inhibitory signal favoring the regulation of the homeostasis in the immune response. To induce inhibitory effects on T cells, CTLA-4 binds PI3K leading to the activation of SHP2 and PP2A phosphatases. The activation of SHP2 determines the dephosphorylation of the CD3ζ chain of the T cell receptor (TCR), limiting its activity (Hebeisen et al., 2013). The binding of CTLA-4 to

PP2A phosphatase diminishes T cell activation inhibiting the Akt phosphorylation. Moreover, it has been demonstrated that the CTLA-4 blockade inhibits the immunosuppressive functions of Treg cells (Walker, 2013). The essential role of CTLA-4 immune checkpoint molecule comes from the *in vivo* study on CTLA4-knockout mice which dead only when were three weeks old due to pancreatitis, myocarditis and the T cell infiltration in the liver, heart, lung and pancreas (Tivol 1995).

PD-1/PD-L1 PD-L2

The PD-1/PD-L1 pathway is essential for the maintenance of the immune homeostasis: PD-1 deficient mice are susceptible to develop lupus-like autoimmune disease (Nishimura et al., 1999) and the inhibition of PD-L1 determines diabetes onset in non obese diabetic (NOD) mice (Wang et al., 2005). Furthermore, PD-1 deficient mice have altered thymic T cell education (Nishimura et al., 2000). Overall these data underlie a key role for the PD-1/PD-L1 axis in the immune tolerance.

PD-1 is mainly expressed on mature T cells but also on B cells, APCs and NK cells. The binding of PD-L1 to its receptor PD-1 on T cells turns off the immune response, counteracting T cell activation signals. PD-1 binds its ligands PD-L1 and PD-L2 and, as for the CTLA-4 signaling, it dephosphorylates PI3K, which inhibits Akt activation leading to a decreased inflammatory cytokine and cell survival proteins production. PD-1 is even strongly expressed on Treg cells and improves their suppressive activity after PD-L1 or PD-L2 binding.

PD-L1 is frequently expressed in cancers serving as a mechanism for tumors to evade the antigenic specific T cell response (Figure 3). Consequently, the PD-1/PD-L1 blockade is nowadays an effective cancer immunotherapy.

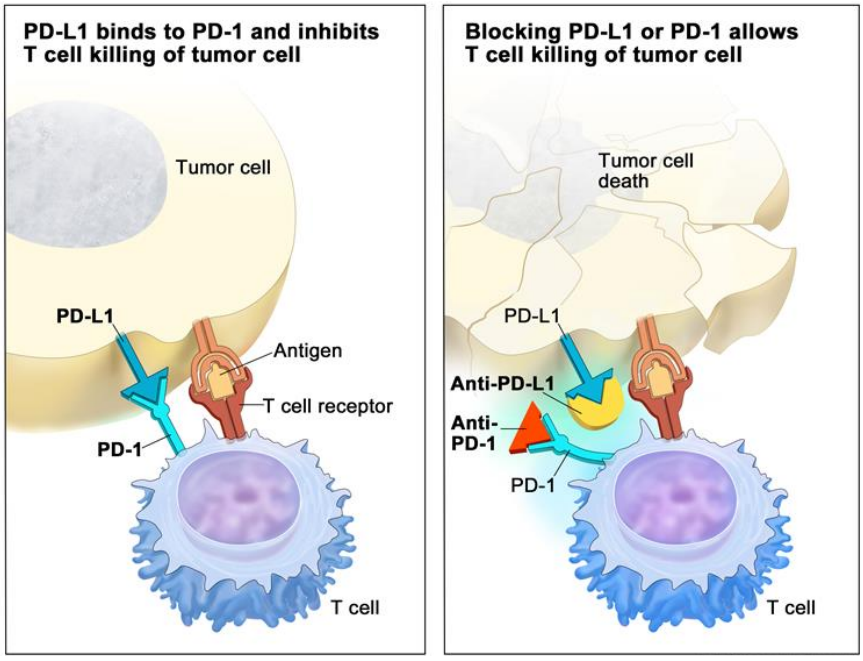


Figure 3. PD-1/PD-L1 immune checkpoint. *Left panel:* the interaction between PD-L1 expressed on tumor cells and PD-1 on T cells lead cancer cells to turn off T cells, deregulating the control of the immune response. *Right panel:* the blocking of the binding of PD-L1 to PD-1 with immune checkpoint inhibitor drugs (anti-PD-L1 or anti-PD-1) allows T cells to kill tumor cells.

LAG-3

Lymphocyte-activation gene 3 (LAG-3) is a protein belonging to immunoglobulin (Ig) superfamily and, similarly to CTLA-4 and PD-1, negatively regulates the immune response, turning-off the T cell response. LAG-3 binds MHC-II impairing the proliferation, the activation and the homeostasis of T cells. Additionally, LAG-3 plays an important role in the induction of the suppressive function in Treg cells (Huang et al., 2004). Although it has been demonstrated that also Lag-3 knockout mice develop autoimmune diseases, these effects are less severe as compared to those developed in CTLA-4 and PD-1 KO mice.

TIM-3

T-cell immunoglobulin and mucin-domain-containing-3 (TIM-3) is expressed on activated T cells, but also on the liver, small intestine, spleen and lung cells. The main ligand of TIM-3 is galectin-9 and their interaction leads to decreased IFNγ production and reduced T cell proliferation (Das et al., 2017).

4-11B

4-11B receptor, also known as CD137, is a member of the tumor necrosis factor receptors (TNFRs) expressed on $CD4^+$ and $CD8^+$ T cells, NK cells, neutrophils and dendritic cells (DCs) (Sanchez-Paulete et al., 2016). 4-11B binds its ligand on activated macrophages and B cells. Differently, from the above-mentioned checkpoint molecules, it is an activating checkpoint, indeed 4-11B receptor bounded to its ligand 4-11B ligand promotes survival and pro-inflammatory processes in $CD8^+$ T cells, bursting the immune response.

The uncontrolled growth of cancer cells results from accumulated abnormalities affecting many of the cell regulatory mechanisms, but it is also dependent on the immune suppression. Thus, the tumor will acquire molecules that are ligands for immune checkpoints and favor in this way the cancer progression.

In the last decade, however, the immunotherapy has focused on the development of molecules that interfere with checkpoints and reactivate the anti-tumor immune response.

1.2. CRC

CRC is the most common cancer in Europe according to the GLOBOCAN data and, despite the significant improvements in screening and treatments, it remains one of the leading cause of tumor-related mortality.

CRC is caused by genetic and environmental factors, which lead to the transformation of normal colon mucosa into invasive cancer (Binefa et al., 2014). The main factors responsible for the development of transformed colon tissue are

the accumulation of genetic and epigenetic alterations; furthermore, over the 70% of CRC cases are related to the lifestyle in which the risk factors are represented by the sedentary lifestyle, alcohol abuse, tobacco and being overweight (Zamani et al., 2018). Additionally, in the last decade, always stronger attention has been given to the role of microbiota in carcinogenesis in approximately 20% of cancers, particularly CRC (Collins et al., 2011).

1.2.1. CRC Pathogenesis

CRC has strong heterogeneity and three are the main molecular and pathological pathways considered for its pathogenesis:

- chromosomal instability (CIN) was proposed by Fearon and colleagues. They demonstrated that the accumulation of numerical or structural mutations lead to the *KRAS* oncogene activation and to the inactivation of the suppressor genes *APC*, *SMAD4*, *TP53* (Fearon and Vogelstein, 1989). The ras protein is activated by the binding of GTP (guanosine 5'-triphosphate), while it becomes inactive when GTP is hydrolyzed to guanosine 5'-diphosphate. Mutations in *KRAS* affect the GTP-binding domain leading to the constitutive activation of ras (McCormick, 1989).

  The *APC* mutation is one of the most recurrent aberrations in CRC being present in 60% of colonic and 82% of rectal cancers. Mutations in the tumor suppressor gene *APC* consist mainly in the truncation of the protein leading to the blocking of the APC binding to β-catenin which suppress the *Wnt*-pathway (Nusse and Clevers, 2017).

  SMAD4, together with *SMAD2*, is involved in the TGF-β signaling pathway and alterations in these genes can cause juvenile polyposis syndrome, which is associated with CRC (Fleming et al., 2013).

  Lastly, allelic loss of *TP53* is frequent during the transition from adenoma to adenocarcinoma. The tumor suppressor gene *TP53* regulates several cellular processes such as cell cycle, apoptosis, and DNA repair. In the context of CRC, it has been recently

demonstrated that p53 mutations, additionally to the role in the adenoma-adenocarcinoma transition, are linked to the lymphatic and vascular invasion in distal CRC. Furthermore, it has been shown that patients with CRC characterized by mutated p53 are more chemo-resistant and showed a poorer prognosis (Li, 2015).

- Microsatellite instability (MSI) causes error accumulation in the process of DNA replication due to germline mutations in the genes accountable for the mismatch repair (MMR), such as *MSH2*, *MSH3*, *MSH6*, *PMS1*and *PMS2* (Nojadeh et al., 2018). Microsatellites are nucleotide repeat sequences whose replication is susceptible to making errors, thus determining genome instability. Several microsatellites are present in genes implicated in CRC such as *MSH3*, *BAX*, *APC*.

  MSI in CRC has a prognostic role, indeed it has been demonstrated in several studies that increased survival takes place in tumor patients who show MMR deficiency in comparison with MMR-proficient tumor patients (Sinicrope et al., 2011).

- The second most common cause of sporadic CRC is the epigenetic pathway caused by dinucleotide hypermethylation in the gene promoter regions and it is referred to as the CpG Island Methylation Phenotype (CIMP). Many CRC shows MSI due to the epigenetic silencing of MLH1 caused by the promoter hypermethylation. This process leads to the accumulation of mutations in other genes involved in colorectal carcinogenesis such as *TGF-β* or *BAX* (Zeinalian et al., 2018). There is not yet a panel of CIMP markers, but Weisenberger and colleagues have defined that the presence of at least three markers between *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and SOCS1 allows defining a tumor CIMP-positive (Weisenberger et al., 2006).

The characterization of the CRC pathogenesis permits to define prognostic markers and more importantly to define new therapeutic targets for CRC treatments.

1.2.2. Gut microbiota

The contribution of gut microbiota in the colorectal carcinogenesis is becoming increasingly clear. The first evidence reporting the involvement of gut microbiota in colorectal carcinogenesis dates back to 1975 in a study reported the presence of less developed colorectal tumors in germ-free rats as compared to the healthy rats (Narisawa, 1975). It has been recently revealed the central role of microbiota also in the cancer treatment; Sivan and colleagues demonstrated that anti-PD-1 or anti-PD-L1 therapy was effective in melanoma-bearing mice thanks to the contribute of *Bifidobacterium longum* and *B. Breve*. Furthermore, the same authors revealed that *Bifidobacteriales* were also found to be strongly represented in the colon of mice with reduced growth of melanoma (Sivan et al., 2015). The positive role of the microbiota in supporting the anti-PD-1 therapy was also observed in patients (Gopalakrishnan et al., 2018; Matson et al., 2018; Santoni et al., 2018). One of the more recent evidence of the impact of gut microbiota in the immunotherapy has been underlined in lung and renal carcinoma patients with an antibiotic-induced dysbiosis that showed compromised efficacy to the anti-PD-1 therapy (Routy et al., 2018).

The gut microbiota covers important functions in the maintenance of the gut homeostasis; furthermore, it represents the natural defending barrier to infections. The presence of a balanced microbiota during our growth is a necessary step for the correct development of the immune response; it has been reported in fact the presence of immune irregularities in germ free mice grown in bacteria-free conditions (Ivanov et al., 2008); additionally, in the past has been already revealed the ability of microbiota to restore the mucosal immune system in germ-free mice (Umesaki et al., 1995).

The gut microbiota can be subdivided depending on its location in the intestinal tract: the *luminal flora* is in the lumen and the *mucosa-associated flora* is constituted by microbes that penetrate the mucosal layer (Sekirov et al., 2010). The murine microbiota is comparable to the human in term of composition (Hugenholtz and de Vos, 2018); therefore the mouse gastrointestinal experimental conditions have a big impact on translational studies.

Increasingly evidence reported alterations in the gut microbiota composition following environmental changes, such as infection, lifestyle changes and the insurgence of cancer. CRC is a totality of tumor cells, stromal cells and a large number of microorganisms; it has been associated with an altered microbiota composition, which is responsible of a state of dysbiosis (Hajishengallis et al., 2012). The main reported bacterial species found to have relevant pro-carcinogenic roles in the gastrointestinal tract during colon cancer development are *Streptococcus Bovis*, *Helicobacter Pylori*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Clostridium Septicum* and *Escherichia Coli*. The pro-carcinogenic roles of microorganisms are represented by virulence factors, inflammation induction, oxidative stress or even the modulation of the host immune response. Microorganisms are able to remodel the microbiota teaching it to become pro-inflammatory and to drive the transformations of epithelial cells; in particular, microorganisms induce epithelial DNA damage and promote the proliferation of the mutated populations (Tjalsma et al., 2012).

However, the mechanisms inducing dysbiosis and the role of dysbiosis as the cause or a consequence of CRC, remain still undisclosed; therefore, further investigation is necessary to bring to light the intricate role of gut microbiota in the context of CRC.

1.2.3. Gut barrier permeability and bacterial translocation

The gastrointestinal tract is a large surface which permits the entrance of the water and the nutrient substances acquired through the food; but at the same time, it constitutes a strict barrier to block the ingress of dangerous and harmful substances and pathogens that pose a threat to our body. This balance is maintained by the collaboration between the structural component of the gut barrier and the molecular interactions that together preserve the intestinal homeostasis (Turner, 2009).

The gut barrier is composed by the mucus layer including the commensal gut microbiota, the secretory immunoglobulins A (sIgA) and the antimicrobial peptides; the layer of epithelial cells includes enterocytes, goblet cells, and Paneth cells that separates the lumen from the lamina propria. The lamina propria is the

site in which reside the immune cells such as T cells, B cells, macrophages, and DCs (König et al., 2016).

The entrance of small molecules, ions, and solutes in the gut takes place in the epithelial cell layer through the presence of the junction complexes. The tight junction proteins connect the epithelial cells and have key roles in regulating the paracellular permeability (Tsukita et al., 2001). Indeed, alteration in the composition of the tight junctions leads to an impaired integrity of the gut barrier and to the risk to develop infective, inflammatory diseases and colon cancer (Soler et al., 1999).

Another important aspect coming from the impaired structure of the gut barrier is represented by the ability of microbial products and bacteria to translocate from the intestinal lumen to the adjacent organs through the circulation (Figure 4). A large body of literature has demonstrated that bacterial translocation occurs in colorectal cancer both in mice (Bråten et al., 2017; Puppa et al., 2011) and patients (Chin et al., 2007).

In this scenario, it is therefore fundamental to assess and monitor gut barrier dysfunction in colorectal cancer (Soler et al., 1999).

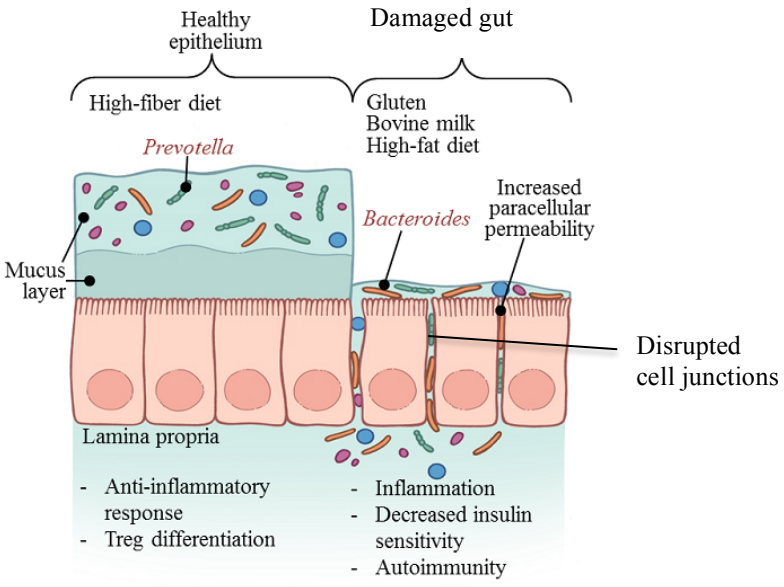


Figure 4. Representative scheme of impaired gut barrier and intestinal permeability. The left panel shows the healthy gut. In the right panel a damaged gut is showed. The cell junctions are destroyed leading to the increased permeability. Microbial products and bacteria can, in this way, invade the near organs through the circulations.

1.2.4. CRC treatments

The conventional therapy for CRC is represented by combined radiotherapy and chemotherapy before surgery; in fact, the surgery is the main treatment for optimizing the chances of healing. The conventional chemotherapeutics used for CRC are 5-FU in combination with Oxaliplatin and the folinic acid Leucovorin which is known as FOLFOX; instead, the FOLFIRI scheme uses 5-FU, Leucovorin and the inhibitor Camptosar.

In this scenario several new targeted drugs have emerged in the last decade:

- Bevacizumab is a partially humanized monoclonal antibody directed against VEGF (Vascular Endothelial Growth Factor). In combination with oxaliplatin it has shown increase progression-free survival (Saltz et al., 2008);
- Cetuximab is a partially humanized monoclonal antibody directed against EGFR (Epidermal Growth Factor Receptor) effective only for the *KRAS* mutated tumors;
- Panitumumab is a humanized monoclonal antibody against EGFR effective in monotherapy or in combination with chemotherapy;
- Ramucirumab is a humanized monoclonal antibody against VEGFR. It acts in combination with chemotherapy;
- Aflibercept is a molecule targeting VEGF which has been demonstrated to increase Overall Survival (OS) in combination with chemotherapy (Lockhart et al., 2012).

Nonetheless, the therapeutic approaches are increasing. Recently, the therapy with the anti-PD-1 monoclonal antibody Nivolumab has been approved for the treatment of metastatic colorectal cancer refractory to the conventional chemotherapeutic drugs (Overman et al., 2017).

The mentioned data reveal that increasingly important steps have been taken in this field, but certainly further molecular and genetic studies are indispensable for the treatment of CRC in the next future.

1.3. B cells

1.3.1. B cell development

B cells are the main actor of the humoral immune response, while T lymphocytes guide the cell-mediated immunity. B lymphocytes are so-called due to their first identification in the bursa of Fabricius, a sac-like lymphatic organ present only in birds and situated dorsal to the cloaca.

Along the maturation process, B lymphocytes acquire the B cell receptor (BCR) specificity. The BCR is a receptor complex composed by a molecule of the Ig family responsible of the antigen binding in association with other accessory molecules such as CD79a (Igα) and CD79b (Igβ). Each BCR recognizes a specific antigen; therefore the organism produces a huge number of different lymphocytes in order to extend the ability of B cells to protect our body.

1.3.2. B-1 B cells

B cells are classified in different subpopulations that develop from specific progenitors. The hematopoietic stem cells (HSC) derived from the fetal liver give origin to the B-1 B cells. The B-1 B cells differ from the majority of B cells: they reside mainly in the pleural and peritoneal cavities, produce IgM against T-independent antigens and are sustained by self-renewal in the periphery (Ghosn et al., 2011). B1-B cells can express the CD5 molecule and during ontogenesis develop early compared to conventional B lymphocytes. Furthermore, the B-1 B cells are independent on the B cell stimulating factors, contrary to the B2-B cells (Scholz et al., 2008).

B1-B cells can be further divided into B-1a e B-1b subsets. B-1a B cells produce natural antibodies and occur in the first line of defense, while B-1a B cells have a role in long-term protection (Alugupalli et al., 2004).

Recent studies have underlined the reduced ability of adult HSC to give origin to B-1a lymphocytes as compared to the fetal HSC. This observation supports the hypothesis that the B-1a B cell population is established in the early stages of life and derives from the lymphatic cells present in the fetal liver (Sawai et al., 2016; Yuan et al., 2012).

1.3.3. B-2 B cells

The majority of B cells originates from the HSC in the BM through a finely orchestrated process and is called B-2 B cells or conventional B cells. The B-2 cells are directed to develop into B cells of the marginal zone (MZ) or into follicular (FO) B cells.

In mice, the MZ B cells stably reside in the compartment at the interface between the red and the white pulp of the spleen. B-2 B cells counteract the pathogens that enter the circulation, guarantying a timely response mediated by the production of low-affinity IgM (Allman and Pillai, 2008). Furthermore, the MZ B cells are able to activate T lymphocytes through the antigen presentation (Attanavanich and Kearney, 2018).

The FO B cells circulate in the lymph nodes and in the spleen. After the antigen encounter and the contact with the T helper cells, that provide co-stimulatory signals, B-2 FO B cells proliferate and differentiate into antibody-producing plasmablasts. Then, the B cells migrate into the follicles and create, with T cells, a germinal centre (GC) where the antibody response is refined through the somatic hypermutation and the isotype switch.

While the development of B-1 B cells is still debated, much more is known about the B-2 B cells development process.

In mice, the Flt3 receptor covers a key role in B lymphopoiesis. Flt3 receptor impairs the capacity to differentiate into megakaryocytes and erythrocytes (Adolfsson et al., 2005). In accordance, mice lacking Flt3L have impaired B cell development (Sitnicka et al., 2002). Another important mediator of B cell development is IL-7. IL-7 acts in a later stage and, also in this case, mice lacking Flt3 do not present a normal B cell population (Peschon et al., 1994).

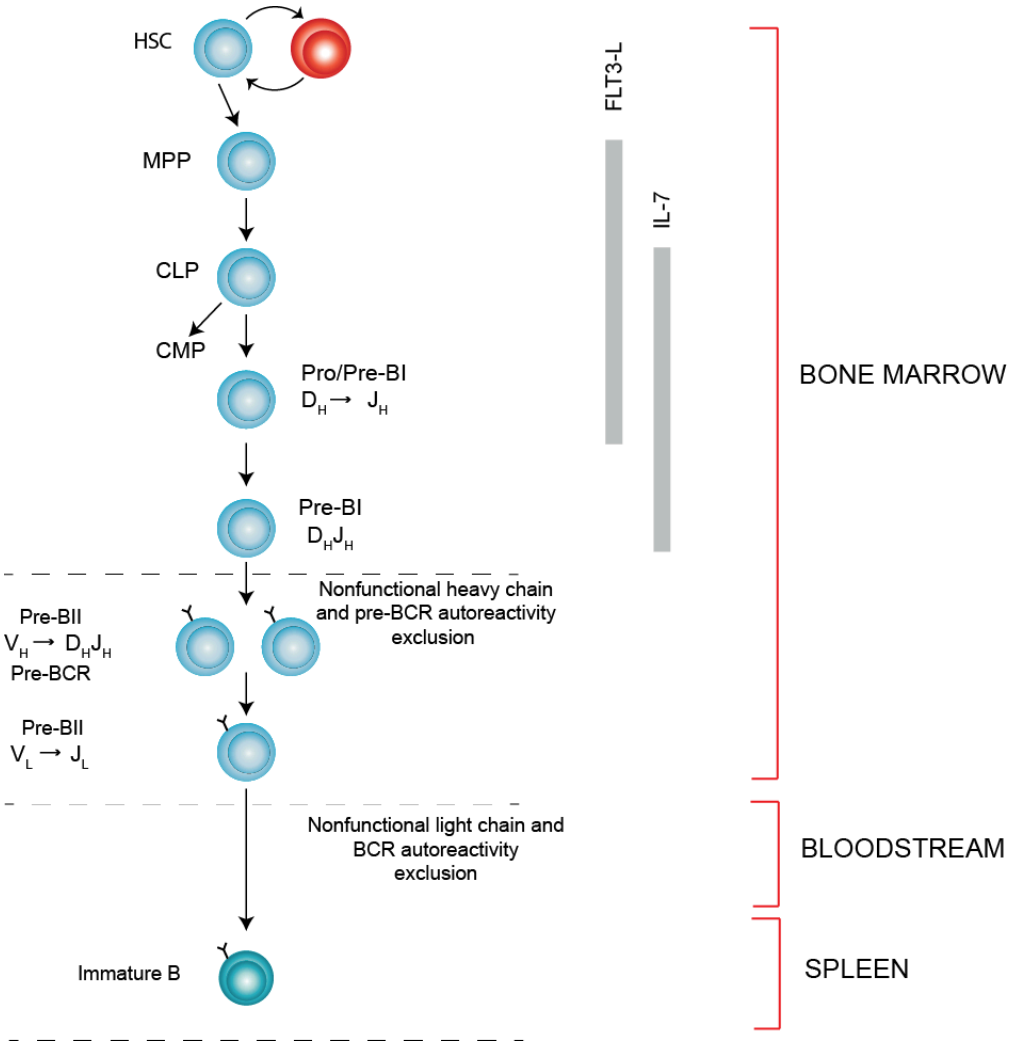


Figure 5. B cell development. B cells origin from the HSC in the BM. Their development is guided by the rearrangement of immunoglobulins and the exclusion of B cells expressing auto reactive BCRs.

The aim of the B cell maturation is the generation of a functional BCR, which is essential for B cell identity. In order to increase the variability of the binding sites, the B lymphocyte genome is prepared through the rearrangement of V, D and J genes at the loci of Ig heavy and light chains (Tonegawa, 1983) and through the nucleotide insertion mechanism at gene junctions (Komori et al., 1993).

The pro-B cell is the first step of B cell development in which the recombinase activating genes 1 and 2 (RAG1/2) join the segments D and J_H at the immunoglobulin heavy (*IgH*) chain locus. Afterward, the rearrangement of V_H to

DJ_H occurs and the resulting V_HDJ_H recombination at *IgH* couples with a surrogate light chain (λ5-Vpre-B) forming the pre-BCR. The pre-BCR is exposed on the cell surface and the signaling activation lead to the reduced expression of the RAG1/2 proteins and to an increasing proliferative signaling in the so defined large pre-B cells (Jung et al., 2006). The RAG1/2 proteins are re-expressed at small pre-B cell stage, leading to light chains rearrangement and the transition of pre-B cells in immature (IMM) BM B cell stage.

At this point, there is the expression of a whole BCR formed by a correct IgM molecule in association with the heterodimer Ig-α/Ig-β that constitutes the signal transmission system of the BCR.

After the complete formation of the BCR, B cells leave the BM and entry the circulation as transitional (TR) B cells. This is the last stage of B cells before becoming one of the two mature pre-immune B-2 pools: the MZ or FO B cell subsets. The maintenance and survival of the mature pre-immune B-2 cells are dependent also on cytokine signals, mainly of those belonging to the B lymphocyte stimulator (BLyS), which is part of the tumor necrosis factor (TNF) superfamily consisting of two additional cytokines, that are fundamental in B cell development: the B cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL); and three receptors: B cell activating factor receptor (BAFF-R), transmembrane activator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). As a matter of fact, it has been demonstrated that the deficiency of both BAFF and BAFF-R is associated with strong reductions in TR and mature B cells (Harless et al., 2001; Warnatz et al., 2009).

1.3.4. Checkpoints of B cell development

The acquired tolerance allows the elimination of immature lymphocyte clones bearing autoreactive antigen receptors (Billingham, et al., 1953). The recombination and the nucleotide insertion processes lead to the formation of autoreactive BCRs. Therefore, mechanisms to eliminate autoreactivity are necessary.

In order to block autoreactivity, different strategies have been highlighted in the literature; one of them leads the cells with autoreactive BCRs to dye. The BCR

signaling in the immature stage can induce continued RAG expression causing light chain rearrangements; therefore another mechanism, known as receptor editing, consists in the editing of the receptor by further VDJ recombination to induce the expression of a not-self reactive BCR (Goodnow et al., 2005).
Accordingly, it has been underlined that the checkpoints of B cell development result altered in autoimmune patients (Meffre and Wardemann, 2008).

1.3.5. B cell activation

B cells activation, generally, need the two-signal paradigm in which the first activation signal occurs after the Ag binding to the BCR (Bretscher and Cohn, 1970). Additionally, B cells need a second activation signal derived via other cells and molecules. Based on this second signal, it is possible to distinguish two categories of responses: the thymus-dependent (TD) and the thymus-independent (TI) responses.
The TD response occurs when, after the BCR ligation, the Ag is internalized, digested and exposed on the B cell surface due to the MHC-II molecule; in this way the protein antigen is presented to the CD4 helper T cells. The TI response can be further divided into two subtypes of responses: the TI-1 response is mediated due to the Toll-like receptors (TLR) expressed on B cell surface; the TI-2 response involves the BCR crosslinking.
B2-B cells, especially the FO B cells, participate in TD responses, while B-1 B cells or the B-2 MZ B cells take part in TI response. Both the TD and TI responses culminate in the antibodies production, but the TD response is more protracted and results in a more robust generation of long-lived antibody-producing plasma cells and memory B cells.
During TI response, antibody-producing plasma cells appear in the splenic extrafollicular regions and mainly produce IgM with low antigen affinity (Hoffman et al., 2016). The TI responses are of short duration, indeed after two weeks, the majority of plasma cells die. In TI response, it has been also highlighted the role of some activated B-1 B cells that produce natural antibodies (Choi et al., 2012). Natural antibodies are polyreactive antibodies, which bind Ag epitopes as well as self-antigens with a restricted set of variable regions. In this

scenario, it has been demonstrated the presence of activated autoreactive B cells in mice prone to develop autoimmune diseases (Herlands et al., 2008).

During TD response substantial numbers of low-affinity IgM producing plasma cells migrate in the splenic extrafollicular regions. Differently, from the TI, the TD response has a longer duration and proliferating B cells and T cells migrate in the so-called GCs, in the lymph nodes and spleen (De Silva and Klein, 2015).

The bidirectional interaction between activated CD4 T cells and antigen-presenting B cells is necessary for the formation of the GC structures. The interaction mechanisms include the CD40-CD40L co-stimulation, MHC-II Ag presentation by B cells and the participation of cytokines (Victora and Nussenzweig, 2011). These interactions induce the assumption of a specific B cell transcriptional program. One of the main upregulated genes is the activation-induced deaminase (AID); it is responsible for the somatic hypermutation (SHM) process in B cells causing mutations in the V region of the Ig. It follows that clonal variants of B cells with different specificities are produced (Gitlin et al., 2014). Despite this process is finely regulated, the formation of autoreactive B cell clones is unavoidable; therefore, GC selective checkpoints exist in both humans and mice (McHeyzer-Williams et al., 2006).

TD response results in the formation of long-lived plasma cells and memory B cells. The former resides in the BM and persists for all the life; the latter is able to rapidly proliferate and are less dependent on T cell for their reactivation (Maruyama et al., 2000).

1.4. IgA

The gut is the most considerable producer of immunoglobulins in our body where the majority of activated B cells differentiate into IgA plasma cells. The mucosal surfaces are continuously exposed to potentially harmful agents and IgA represent the first line of defense at these sites (Cerutti, 2008).

Mucosal plasma cells produce secretory dimeric and polymeric IgA (pIgA); the latter generates from the interaction of IgA monomers with a joining chain (J chain), which promotes the interaction with the pIg receptor (pIgR) expressed on secretory epithelial cells (Mostov and Deitcher, 1986). This process results in the

passage of sIgA across the epithelial barrier and their entrance into mucosal surfaces (MacPherson et al., 2008). sIgA perform the bacterial shipment to the epithelial microfold cells (M cells), that are localized adjacent to the Peyer's patches. M cells implement the transport of the microorganisms to the lamina propria where the immune response takes place (Mabbott et al., 2013). IgA is not able to activate the complement system, therefore sIgA induce pathogen elimination without triggering the inflammatory process.

Differently, from the sIgA, the circulating or systemic IgA in humans are mainly monomeric; while in mice, the polymeric IgA are predominant. Systemic IgA binds receptors expressed by several kinds of cells, such as DCs, macrophages, hepatic and renal cells. The binding to the myeloid Fcα receptor for IgA (FcαRI) has been reported to potentially generate a second line of defense against the gut bacteria entry in the venous circulation (Otten and Van Egmond, 2004).

Differently from mice, in humans exist two subclasses of IgA: IgA1 and IgA2. Two genes Cα1 e Cα2, located on chromosome 14, encode their heavy chains (Woof and Ken, 2006). The main structural difference between IgA1 and IgA2 is that IgA2 has a shorter hinge region that confers stronger resistance against degradation by bacterial proteases (Flanagan et al., 1984).

IgA1 and IgA2 are differently distributed in the body: the IgA1 mainly represents the circulating IgA, whereas on mucosal surface we have both IgA1 and IgA2. In particular, IgA2 are predominant in intestinal sites abundantly colonized by the microbiota, such as the distal tract.

1.4.1. IgA class switching mechanisms

The process through which B cells express IgA, the most abundant antibody isotype in our body, is the IgA class switching.

The antibody diversity required for effective humoral responses is generated by antigen-independent mechanisms in the BM such VDJ recombination, and by antigen-dependent mechanisms such as SHM and the class switch recombination (CSR), when B cells migrate from BM to the secondary lymphoid organs. The class switching process substitutes the *IgH* constant region genes Cμ and Cδ codifying IgM and IgD, with Cα, Cγ or Cε which respectively encode for IgA,

IgG, and IgE isotype antibodies.

The class switching is guided by the switch (S) regions, located upstream of the C_H gene. When B cells are activated, the C_H gene transcription starts, making the S region a substrate for AID that deaminates cytosine residues generating several DNA lesions. The fusion of the DNA breaks allows the juxtaposing of V_HDJ_H to Cα producing a DNA sequence encoding the IgA protein.

The processes occurring in the GC are in general dependent on the interaction between B cells and CD4 T cells. The CD40/CD40L axis is one of the main mechanisms for B/CD4 T cells interaction. The T cells express CD40L, a member of the TNF family that binds CD40 on the B cells surface leading to the activation of the IκB kinase enzymatic complex (IKK). IKK mediates the phosphorylation of inhibitor of NF-κB (IκB), which induces the retention of nuclear factor-κB (NF-κB) in a cytoplasmic inactive form. The IκBα phosphorylation by IKK is followed by the degradation of IκBα that allows the NF-κB translocation into the nucleus (Siebenlist et al., 2005). At this point NF-κB leads to the activation of promoters of key B cells genes such as Iγ and Iε promoters. Beyond activating IgG and IgE class switching, NF-κB is also essential in the IgA class switching. It has been in fact demonstrated that NF-κB deficient mice show impaired IgA class switching process.

Another important T cell-dependent mechanism for IgA class switching is in response to the transforming growth factor β1 (TGFβ1) that activates the promoters of Cα genes. In this case, TGFβ1 engages TGFβ receptor (TGFβR) and low levels of TGFβ1 start the Cα gene transcription. With regard to this process, Rubstov and colleagues showed the insurgence of autoimmune disease in mice lacking TGFβ1 (Rubtsov and Rudensky, 2007). The body geographical localization of the described T cell-dependent IgA class switching mechanisms reside mainly in the intestinal Peyer's patches, where DCs present the Ag to CD4 T cells leading to the production of IgA inducing cytokines such as TGFβ1, IL-6, and IL-10.

The anatomical localization of T cell-independent IgA production is the lamina propria as revealed by evidences that point out the presence of IgA CSR not only in lymphoid follicles but also in extrafollicular areas, such as subepithelial areas

(Xu et al., 2007). The T independent IgA class switching occurs in presence of LPS or other molecules such as polysaccharides that lead the activation of TLR or the BCR. Furthermore, in this context, also DCs and epithelial cells cover important roles: during the Ag presentation to B cells, DCs release class switching inducing factors, such as BAFF and APRIL. In addition, epithelial cells release several molecules, for example, IL-10 and TGFβ1 that induce IgA CSR. In humans, in addition to IL-10, APRIL is involved in the induction of IgA2 suggesting the relevant role of epithelial cells in the IgA induction.

Despite a multitude of mechanisms inducing IgA class switching has been described, there are still IgA function and mechanism to clarify. Recently, several works reported the IgA involvement in the tumor context (Mion et al., 2017; Shalapour et al., 2015; Welinder et al., 2016). In the study of Mion and colleagues, they revealed the presence of elevated IgA levels in the culture supernatants of untreated and stimulated splenic and peritoneal B lymphocytes of the colorectal cancer mouse model $Apc^{Min/+}$ as compared to the WT mice. Furthermore, the authors found also that IgA levels were also increased in the $Apc^{Min/+}$mouse sera than in those of WT mice, indicating a strong role of the tumor microenvironment in the priming toward IgA production. This intriguing involvement of IgA response in the adenomatous transformation may be crucial for the generation of specific therapies, but further exploration is required.

1.5. Immunosuppressive B cell subsets

The key role of B cells as positive regulators of the immune response has historically been attributed to their ability to produce antibodies; furthermore, B cells are also able to act as APCs and to produce cytokines. In the last decade, a plentiful literature has highlighted the presence of immunosuppressive B cell subsets characterized by the ability to switch off the immune response in the context of autoimmune diseases and cancer.

1.5.1. B regulatory cells

The first evidence of the presence of a B cell subset with regulatory functions

comes from studies on mice with experimental autoimmune encephalomyelitis (EAE) showing that the inflammatory state could expand a population of IL-10-producing B cells (Fillatreau et al., 2002). In the following years, an increasing number of evidences were collected reinforcing the regulatory characteristics of these B cells that were called Breg (Mauri and Bosma, 2012; Vitale et al., 2010). Until now, several studies reported different groups of phenotypic markers for the Breg as reported in Table 1 (Rosser and Mauri, 2015), but the definition of specific markers for the Breg remains a debated field, in both mice and humans. In fact, differently from Treg cells that are unequivocally identified through the expression of the transcription factor FoxP3, the main problem of Breg cells is the lacking of specific markers, therefore studies focused on identifying Breg markers are needed (Tonon et al., 2018. Submitted).

Table 1. Different Breg Cell Subsets

Type of Breg Cell	Mouse	Human	Key Features	Reference
T2-MZP cells	$CD19^+CD21^{hi}$ $CD23^{hi}CD24^{hi}$	–	found in spleen, produce IL-10, induce Treg cells, and suppress effector $CD4^+$ and $CD8^+$ T cells	Blair et al., 2009; Carter et al., 2011; Evans et al., 2007; Schioppa et al., 2011
MZ cells	$CD19^+CD21^{hi}$ $CD23^-$	–	found in spleen, produce IL-10, induce Treg cells, and suppress effector $CD4^+$ and $CD8^+$ T cells	Bankoti et al., 2012; Gray et al., 2007; Miles et al., 2012
B10 cells	$CD5^+CD1d^{hi}$	$CD24^{hi}CD27^+$	found in spleen (mice) and blood (humans), produce IL-10, and suppress effector $CD4^+$ T cells, monocytes, and DCs	Horikawa et al., 2013; Iwata et al., 2011; Matsushita et al., 2010; Yanaba et al., 2008
Plasma cells	$CD138^+$MHC-11^{lo} $B220^+$	–	found in spleen, produce IL-10 and IL-35, and suppress NK cells, neutrophils, and effector $CD4^+$ T cells	Neves et al., 2010; Shen et al., 2014
Tim-1^+ B cells	Tim-1^+CD19^+	–	found in spleen (mice), produce IL-10, and suppress effector $CD4^+$ T cells	Ding et al., 2011; Xiao et al., 2012
Plasmablasts	$CD138^+CD44^{hi}$	$CD19^+CD24^{hi}$ $CD27^{int}$	found in dLNs (mice) and blood (humans), produce IL-10, and suppress DCs and effector $CD4^+$ T cells	Matsumoto et al., 2014
Immature cells	–	$CD19^+CD24^{hi}$ $CD38^{hi}$	found in blood and at site of inflammation, produce IL-10, induce Treg cells, suppress Th1 and Th17 cells, suppress virus-specific $CD8^+$ T cell responses, are defective in patients with SLE and RA, and support iNKT cell homeostasis	Blair et al., 2010; Bosma et al., 2012; Das et al., 2012; Flores-Borja et al., 2013
Br1 cells	–	$CD19^+CD25^{hi}$ $CD71^{hi}$	found in blood and produce IL-10 and IgG4	van de Veen et al., 2013

Table 1. Breg phenotypic classification. Main B cell subsets and their phenotypic characterization in mice and humans (Rosser and Mauri, 2015).

Although the phenotype of Breg remains a topic of discussion, several B cells subsets have been classified for their immunosuppressive activity that can be

mediated by a variety of mechanisms that target many immune cells such as DCs (Matsushita et al., 2010), macrophages (Wong et al., 2010) and Th cells (Tian et al., 2001). The most effector function of Breg is the release of the anti-inflammatory cytokine IL-10, however other cytokines like TGFβ or IL-35 exert the immunosuppressive function inhibiting T cell proliferation and cytokine production (Mauri et al., 2003). Additionally to the cytokine release, Breg act also in a cell contact-dependent manner. CD40/CD40L axis has been reported to be one of the main mechanisms used to inhibit T cells. Other co-stimulatory molecules that take part in cell contact-dependent suppressive functions of Breg are CD80 and CD86; the interaction with their inhibitory ligands, such as CTLA-4, on target cells, inhibits T cell proliferation and induce Treg recruitments (Fife and Bluestone, 2008).

1.5.2. Tumor evoked B cells

Cancer is able to evade the immune response taking advantage of immunosuppressive cells. Between them, myeloid-derived suppressive cells (MDSCs) and Treg cover relevant roles (Olkhanud et al., 2009; Youn et al., 2008).

Olkhanud and colleagues have recently described a new B cells subset designated as tumor evoked Breg (tBreg) and characterized by the constitutive expression of active Stat3 and B7-H1^{High} CD81^{High} CD86^{High} CD62L^{Low} IgM^{Int}. The authors revealed a key role of tBreg in the development of metastases in the mouse model of breast cancer 4T1. They demonstrated the ability of tBreg to induce the conversion of resting CD4 T cells into Treg in a TGFβ dependent way, favoring metastatic progression. On the contrary, the authors underlined that the absence of tBreg does not allow the metastatic process of 4T1 tumors due to the weak conversion of Treg (Olkhanud et al., 2011). From this study, a very potential role has been attributed to tBreg that can be regulated to block cancer metastases.

More recently, Bodogai and co-workers underlined another mechanism used by tBreg for inducing the metastatic spreading (Bodogai et al., 2015). The authors demonstrated that MDSCs have limited suppressive function in the 4T1 breast cancer mouse model unless they are educated by tBreg. The authors unveiled the

mechanism responsible for the MDSC education by tBreg that is dependent on TGFβR1/ TGFβR2 signaling; in fact in absence of tBreg and in presence of TGFβR1 deficient MDSCs tumor-bearing mice did not develop metastases and MDSCs do not efficiently produce reactive oxygen species (ROS) and nitric oxide (NO) to suppress T cells.

Overall the reported data highlight the presence of a new subset of immunosuppressive B cells that are essential regulators of immunosuppression and potential inducers of metastatic progression.

1.5.3. Killer B cells

The production of the anti-inflammatory cytokines IL-10 and TGFβ, above described, is one of the mechanisms of the immune regulation implemented by B cells. Furthermore, another mechanism of immune regulation is the activation-induced cell death (AICD) mediated by ligands that induce death in target cells (Green, et al., 2003). The ligands include Fas ligand (FasL), tumor necrosis factor related apoptosis-inducing ligand (TRAIL), PD-L1 and PD-L2 (Alderson and Lynch, 1998). Many lines of evidence reported the presence of a B cell subset showing killer function: these cells mainly express FasL through which induce cell death (Lundy and Boros, 2002), but in the literature it has also been reported the expression of TRAIL, PD-L1 and PD-L2 on activated B cells (Zhong et al., 2007); therefore these cells are called killer B cells (Lundy, 2009).

In the tumor context, the expression of death ligands by tumor cells represents a way to evade immune surveillance. The expression of FasL on B cells was reported in aggressive forms of lymphoma and in B chronic lymphocytic leukemia (B-CLL) patients (Mullauer et al., 1998; Tinhofer et al., 1998), associating FasL expression with poor prognosis; while, B cell malignancies that express lower FasL levels result less aggressive (Grüllich et al., 2003).

In light of these data, the inhibition of death ligands on B cells could have potential clinical implications.

1.5.4. Tumor infiltrating B cells

The contribution of immune cells in tumor progression is now well known (Pardoll, 2015). Immune cells involved in the tumor biology are leukocytes of both myeloid and lymphoid origins, already present in the tissue or recruited from the circulation due to the state of chronic inflammation established by the tumor. These immune populations can be stimulated by the microenvironment to either suppress or favor tumor growth. In the second case, they can be reprogrammed by tumor-released soluble factors to create a tolerogenic environment and to sustain pro-tumor processes. The progress in the study of the immune microenvironment, has mainly highlighted the role of tumor-infiltrating T cells (TIL-T) in the inhibition of the anti-tumor immune response (Santoiemma and Powell, 2015). While the role of B cells is less well understood. B cells are the second most abundant tumor-infiltrating lymphocyte (TIL-B) and their role in the tumor context is debated: on one hand, they can inhibit tumor development through the production of tumor-reactive antibodies, promoting tumor killing by NK cells, phagocytosis by macrophages, and the priming of $CD4^+$ and $CD8^+$ T cells. On the other hand, B cells can promote tumor development through the production of autoantibodies and tumor growth factors (Yuen et al., 2016).

Several recent works reported the presence of tumor-promoting TIL-B: Sholapur and colleagues revealed the presence of immunosuppressive plasmocytes that express IgA, IL-10, and PD-L1 that conferred resistance to oxaliplatin treatment in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. The authors confirmed that the elimination of these cells allows the eradication of oxaliplatin-treated tumors (Shalapour et al., 2015).

In the second work, the authors demonstrated the ability of TIL-B to favor colorectal cancer progression in MIR15A and MIR16-1 knockout mice treated with AOM/DSS. Most of the B cells in the tumor were IgA^+ and expressed high levels of PD-L1, IL-10, TGFβ and repressed the proliferation of CD8+ T cells (Liu et al., 2018).

In light of the reported data, the target of specific B cell subsets may be of therapeutic value in cancer.

1.6. Myeloid derived suppressor cells

During the normal hematopoiesis, immature myeloid cells differentiate into mature myeloid cells. On the contrary, in several pathological conditions such as cancer, infections, sepsis or traumas, a block of the myeloid maturation process occurs, leading to the generation of a population with immunosuppressive functions called MDSCs (Figure 6). MDSCs accumulate in the peripheral blood, spleen, liver, lungs, and tumor (Alicea-torres and Gabrilovich, 2018); furthermore, they can also maintain the ability to differentiate into mature cells *in vivo* and *in vitro* with cytokine stimulation (Bronte et al., 2000).

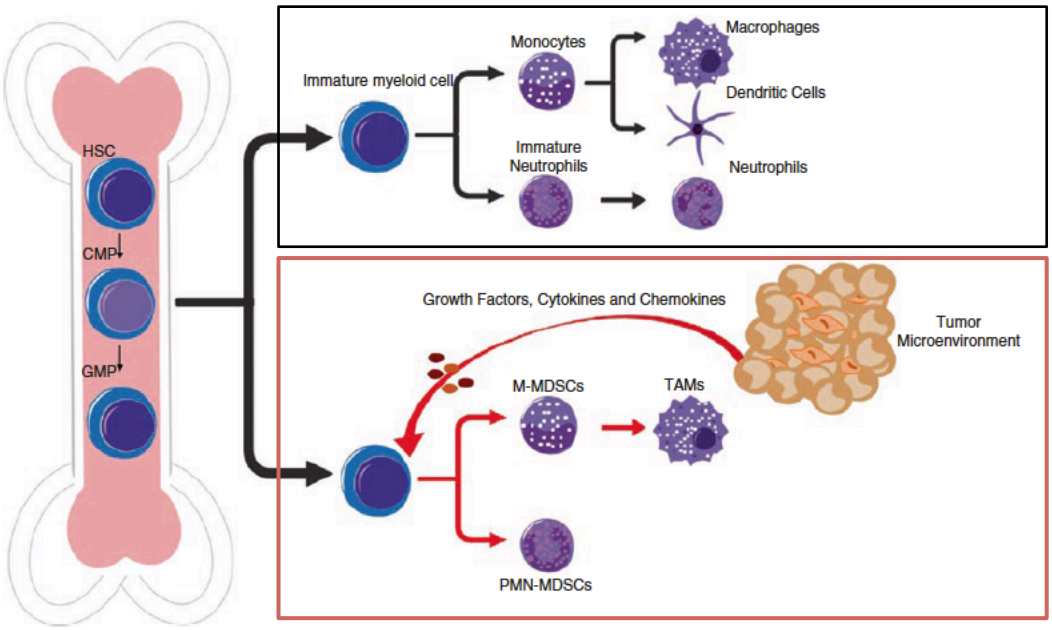


Figure 6. Origin of myeloid derived suppressor cells. In the bone marrow, haematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs) until they become immature myeloid cells (IMCs). IMCs, in normal conditions, migrate to peripheral organs and differentiate into macrophages, dendritic cells or granulocytes *(black box)*. In pathological conditions, such as tumor, the factors released in the microenvironment, induce the accumulation of immature monocytes and neutrophils known as M-MDSCs and PMN-MDSCs, which acquire immunosuppressive functions. M-MDSCs can also differentiate to tumor-associated macrophages (TAMs) that also act to suppress anti-tumor immune responses *(red box)* (Alicea-torres and Gabrilovich, 2018).

MDSCs are now classified as a heterogeneous population of immature myeloid cells with strong immunosuppressive activity associated with cancer progression and correlated with poor prognosis and survival in cancer patients (Gabrilovich and Nagaraj, 2009; Solito et al., 2014).

1.6.1. MDSC phenotype

In mice, MDSCs are characterized by the co-expression of the myeloid lineage differentiation markers CD11b (αM-integrin) and glutathione reductase (Gr-1) (Talmadge and Gabrilovich, 2013). Antibodies binding Ly6G and Ly6C, two different epitopes of Gr-1, allowed to distinguish two main subsets of MDSCs: the granulocytic or polymorphonuclear (G-MDSCs or PMN-MDSCs) and the monocytic (M-MDSC) populations. The PMN-MDSCs are characterized as $CD11b^{+}Ly6G^{+}Ly6C^{low}$, while the M-MDSC subset is defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$. In addition to these subsets of MDSCs, it is now under study a population, representing less than 5% of total MDSCs, that is composed of myeloid progenitors and precursors, but it is not still well characterized, therefore further investigations are needed.

Furthermore, Jayakumar and Bothwell revealed the presence of a further MDSC population in the spleen of Apc^{Min} mice, expressing an intermediate level of Ly6G and Ly6G makers ($CD11b^{+}Ly6G^{lo}Ly6C^{int}$) and therefore defined "intermediate MDSCs" (I-MDSCs) (Jayakumar and Bothwell, 2017). This population expressing intermediate levels of Gr-1 ($CD11b^{+}Gr^{-}1^{int}$) was already been identified in other implanted tumor models (Dolcetti et al., 2010; Marigo et al., 2010).

PMN-MDSCs and M-MDSCs are differently distributed based on cancer types and they use different immunosuppressive mechanisms (Youn et al., 2008).

In humans, MDSC has been mainly studied in the peripheral blood mononuclear cell (PBMC) portion and the absence of a homologous marker to Gr-1 made difficult the direct correspondence between murine and human MDSCs. Nowadays, human PMN-MDSCs are identified as $CD11b^{+}CD14^{-}CD15^{+}$ or

$CD11b^{+}CD14^{-}CD66b^{+}$, while M-MDSCs are defined as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ (Bronte et al., 2016). Also in humans, there is a small population called early-stage MDSCs (e-MDSCs) composed of more immature cells. These subset includes a mix of MDSCs defined as Lin^{-} (including CD3, CD14, CD15, CD19 and CD56) $HLA\text{-}DR^{-}CD33^{+}$ (Alicea-torres and Gabrilovich, 2018).

1.6.2. Mechanisms of MDSC mediated immune suppression

The phenotypic characterization is not sufficient to define myeloid cells as MDSCs, but their ability to suppress effector cells of the immune system has to be assessed. A plentiful literature has highlighted the ability of MDSCs to block the anti-tumor immune response: MDSCs are able to suppress T cell activation and proliferation (Condamine and Gabrilovich, 2011), to impair NK cell cytotoxicity (Liu et al., 2007), to induce Treg (Huang et al., 2006) and skew macrophages to a tumor-promoting M2 phenotype (Sinha et al., 2007a).

They use the increased production of inducible nitric oxide synthase (iNOS) and arginase1 (Arg1), the release of big amounts of ROS and peroxynitrite (PNT), the production of immunosuppressive cytokines and the activity of enzymes involved in the block of T cell effector functions (Figure 7).

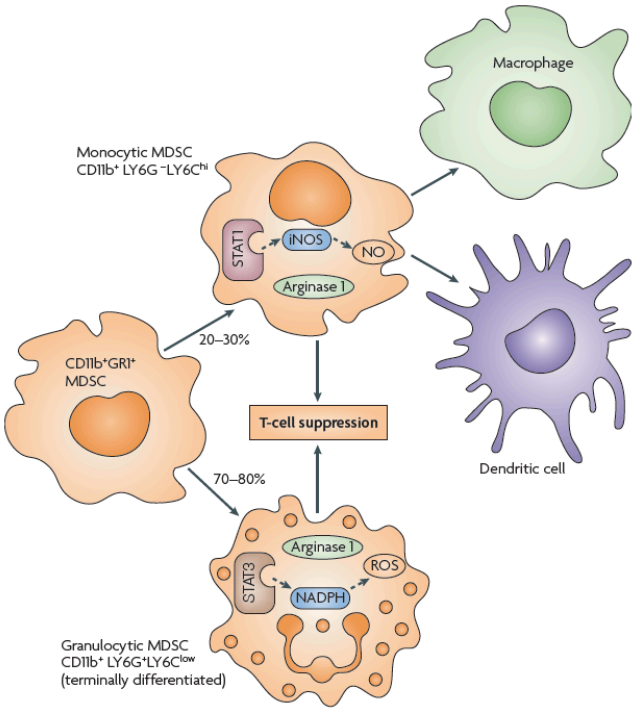


Figure 7. MDSC suppressive mechanisms. Different MDSC subsets might use distinct mechanism to suppress T cells. The granulocytic subset has elevated STAT3 and NADPH activity resulting in a rich production of ROS and in particular of PNT. The monocytic subset shows high expression of STAT1 and iNOS and produce high amounts of NO. Both subsets express increased levels of Arg1 (Gabrilovich and Nagaraj, 2009).

The L-arginine metabolism is strongly involved in the immunosuppression activity of MDSCs. L-arginine is the substrate of both iNOS and Arg1. Arg1 converts L-arginine, an essential amino acid required for T cell proliferation, into L-ornithine and urea determining the depletion of L-arginine in the tumor microenvironment. The scarcity of L-arginine induces the reduced expression of the TCR ζ chain limiting the ability of T cells to mediate the anti-tumor immunity. iNOS generates NO that reacts with superoxide-generating PNT. In addition to the Arg1 effects, also the production of ROS and PNT affects TCR inducing the nitration and nitrosylation of the receptor. This mechanism leads to the impairment of the $CD8^+$ T cell antigen interactions due to the alteration of the Ag binding sites.

In addition to these main mechanisms of immunosuppression, MDSCs produce

immunosuppressive cytokines such as IL-10 and TGFβ to inhibit T cell activation and cytotoxic T cell response (Sinha et al., 2007).

Another mechanism to deplete nutrition factors for T cells is the production of several enzymes: cyclooxygenase 2 (COX-2) has been implicated in the induction of Arg1 in MDSCs (Rodriguez et al., 2005); furthermore indoleamine 2,3-dioxygenase (IDO) has been reported to inhibit T cell activity and to induce T cell apoptosis (Yu et al., 2013).

In this scenario, it has been recently reported that MDSCs can upregulate PD-L1 expression that results in the induction of T cell death through the binding to PD-1 expressed on T cells (Lu et al., 2016).

Although many studies are focused on the identification of the mediators involved in MDSC immunosuppression, further investigations are needed to better clarify the intricate network occurring in the tumor-mediated immunosuppression.

1.6.3. MDSC accumulation and activation

The proposed model for MDSC accumulation requires two signals: the first one blocks the terminal differentiation of the cells leading to their expansion; while the second signal induces the activation of the immature myeloid cells allowing them to become immunosuppressive (Condamine and Gabrilovich, 2011). The two different steps required for the accumulation and the activation of MDSCs are directed by a variety of transcription factors and mediators.

The signal transducer and transcription activator 3 (STAT3) is responsible for MDSC accumulation, in fact, it is has been demonstrated that tumor-bearing mice treated with STAT3 inhibitors had reduced MDSC levels (Condamine et al., 2015). In addition to its role in the accumulation of MDSCs, STAT3 has been also studied as a regulator of MDSC differentiation. Kumar and colleagues reported that M-MDSCs differentiate into TAMs in response to the down-regulation of the activity of STAT3 (Kumar et al., 2016).

Another regulator of MDSC accumulation is the IFN regulatory factor 8 (IRF8); its absence has been correlated with increased MDSC levels in tumor and spleen; on the contrary, its overexpression induces a reduction of MDSC accumulation in tumor-bearing mice (Waight et al., 2013).

Also, the absence of the transcription factor CAAT-enhancer-binding protein beta (C/EBPβ) has been associated with low frequency of MDSCs in tumor-bearing mice, suggesting a role of C/EBPβ in the regulation of MDSC expansion (Marigo et al., 2010). Furthermore, the same role of C/EBPβ has been also attributed to the NLRP3, an intracellular sensor associated with the inflammasome. $Nlrp3^{-/-}$ mice showed lower MDSC levels as compared to the WT mice (Van Deventer et al., 2010).

As above introduced, after their accumulation, MDSCs need a second signal for acquiring immunosuppressive properties. This step is mediated by pro-inflammatory molecules released by the tumor, that activate several signaling pathways. Between them, NF-κB, STAT1 and STAT6 are strongly involved in regulating MDSC activation.

The binding of the tumor produced IL-1β or TNF-α to the TLRs, induces the MDSC activation through NF-κB leading to the production of the immunosuppressive factor PNT (Tu et al., 2008).

Furthermore, the MDSC immunosuppressive activity is induced through the upregulation of iNOS and Arg1. The upregulation of these enzymes is mediated by the activation of STAT1 due to IFNγ stimulation (Kusmartsev and Gabrilovich, 2005).

STAT6 is another transcription factor that regulates the immunosuppression function of MDSCs. The STAT6 signaling is activated through the binding of IL-4 and IL-13 to the receptor IL-4Rα that induces Arg1 expression and TGF-β, which robustly inhibits T cell proliferation (Bronte et al., 2003).

The precise understanding of the molecular mechanisms involved in MDSC activation and functions is crucial for the identification of potential therapeutic approaches to block the immunosuppression process mediated by MDSCs in cancer.

1.6.4. Targeting MDSCs

Since that MDSCs are one of the more aggressive cellular components towards the immune system and one of the main causes of the immune tolerance,

numerous ongoing studies are directed to identify effective strategies that allow the elimination of this population or the modulation of its suppressive functions.

Several chemotherapeutic drugs had positive effects in tumor-bearing hosts: many studies reported that gemcitabine, 5-fluorouracil, cisplatin, and doxorubicin were able to eliminate MDSCs and to induce tumor regression (Alizadeh et al., 2014; Suzuki et al., 2005; Vincent et al., 2010). The elimination of MDSCs was also obtained using a TRAIL-R agonist; insofar it was reported that TRAIL-R regulates MDSC survival (Dominguez et al., 2017).

Furthermore, the use of small pharmacological molecules has given satisfactory results: the COX-2 inhibitors reduce the expression of Arg1, while the phosphodiesterase 5 (PDE5) inhibitors and the NO-realizing aspirin decrease the expression of both Arg1 and iNOS, contributing in this way to the activation of T cells and to the reduction of the tumor progression (Noonan et al., 2014; De Santo et al., 2005; Sinha et al., 2007b; Talmadge et al., 2007).

Another promising approach to contain the immunosuppressive functions of MDSCs is the promotion of their differentiation into mature myeloid cells. The *all trans* retinoic acid (ATRA) was used *in vivo* and *in vitro* and showed great results in inducing the MDSC differentiation into DCs and macrophages, reducing the MDSC expansion (Kusmartsev et al., 2003; Mirza et al., 2006).

2. AIMS OF THE THESIS

CRC is the third most commonly diagnosed cancer worldwide according to the GLOBOCAN data and, despite the significant improvements in screening and treatments, it remains one of the leading causes of tumor-related mortality. Accordingly, the development of new therapeutic strategies is crucial. Considering the growing importance of the tumor macroenvironment in the promotion of tumor growth and invasion to distal organs, new therapeutic options for CRC could result from the characterization of the interaction between the immune system and the tumor TME.

A peculiar involvement of a systemic IgA response in the colon adenomatous transformation has been recently highlighted and requires further exploration (Mion et al., 2017). Due to the fundamental role of IgA in protecting mucosal surfaces, little effort has been made to understand how the systemic IgA system works and how it can be regulated. In particular, the meaning of the IgA skewing and the mechanisms that are at the basis of its modulation are still not described.

Taking advantage of several models of mouse CRC, this thesis aims to understand if the observed IgA skewing constitutes a common feature of all CRCs and which are the roles of IgA in CRC progression. This work will also investigate the possible correlation between the altered intestinal permeability associated with the colon adenomatous transformation and the increased IgA production.

Then, moving to a cell-cell contact context, I decided to investigate whether the large accumulation of MDSCs in the tumoral environment can affect the function of B cells. In the specific, I aimed to investigate if there is a shift towards regulatory B cells but also an IgA skewing and whether the MDSC population drives these processes.

3. RESULTS

3.1. Do elevated IgA levels and CRC go hand in hand?

3.1.1. Elevated IgA levels are peculiar of CRC

Recently, several works reported the IgA involvement in the tumor context (Mion et al., 2017; Shalapour et al., 2015; Welinder et al., 2016; Xu et al., 2017). Mion and colleagues revealed the presence of elevated IgA levels in the supernatants of untreated and stimulated splenic and peritoneal B lymphocytes of the CRC mouse model $Apc^{Min/+}$, compared to the WT counterpart. Furthermore, the authors found high levels of IgA in the sera of $Apc^{Min/+}$ mice, suggesting a strong role of the tumor microenvironment in the priming toward IgA production. This intriguing involvement of an IgA response in the adenomatous transformation may be crucial for the generation of specific therapies, but further exploration about the meaning of the IgA skewing and the mechanisms at the basis of its modulation are required.

3.1.2. IgA skewing is confirmed in the $Cdx2^{Cre}Apc^{flox/flox}$ CRC tumor model

Starting from the evidence mentioned above, in this work we wanted to further reinforce the data published by Mion and colleagues taking advantage of the $Cdx2^{Cre}Apc^{flox/flox}$ mouse model (Hinoi et al., 2007). Since the *Cre* recombinase is under the control of the gut-specific *Cdx2* transcription factor, in this mouse the *Apc* gene is deleted only in the intestinal epithelium and, therefore, the genetic manipulation is not systemic. In this way we could exclude that the altered level of IgA was due to the mutation itself and not due to the insurgence of CRC. A schematic representation of the mechanism is reported in figure 8.

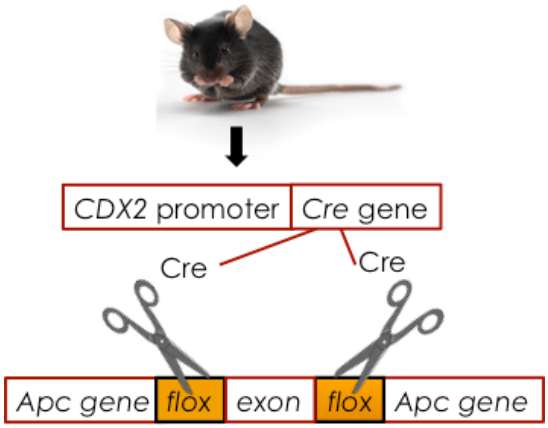


Figure 8. *Cdx2*^{Cre}*Apc*$^{flox/flox}$ mouse model. The gene *Apc* is floxed and excised by the *Cre* recombinase, driven by the gut-specific *Cdx2* transcription factor.

Serum IgA, IgG and IgM levels were quantified and the analyses confirmed the previous data obtained with the Apc$^{Min/+}$ mouse model: serum IgA levels were significantly upregulated in the Cdx2^{Cre}Apc$^{flox/flox}$ tumor-bearing mice compared to the WT control mice. Instead, IgG and IgM levels were not significantly different, resulting comparable between the two groups (Figure 9).

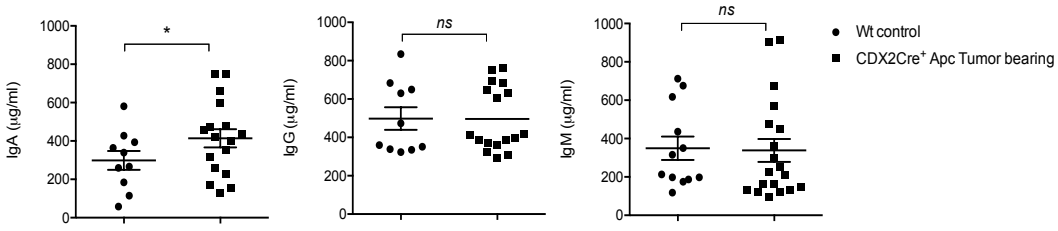


Figure 9. IgA is increased in Cdx2^{Cre}Apc$^{flox/flox}$ mouse model. IgA, IgG and IgM levels were quantified by ELISA in sera of Cdx2^{Cre}Apc$^{flox/flox}$ tumor-bearing and WT mice.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

The *Apc* tumor suppressor gene plays a key role in the transduction of the Wnt-signaling pathway. Since in literature it has been also demonstrated that the

defective Wnt signaling characterizing $Apc^{Min/+}$ mice influences the development and differentiation of several tissues, including haematopoietic tissues (Coletta et al., 2004; You et al., 2006), with our data we can definitely exclude that the previous observed data were the result of the *Apc* mutation.

3.1.3. The sole IgA skewing is peculiar of the $Apc^{Min/+}$ CRC tumor model

In the previously described work, Mion and collaborators reported also that, compared to the WT condition, $Apc^{Min/+}$ splenic B lymphocytes presented traits of an IgA skewing. In order to assess whether this result was peculiar of the splenic compartment, I decided to analyse the IgA skewing also in B cells purified from the MLNs and iLNs of $Apc^{Min/+}$ mice. B cells purified from MLNs revealed an increased release of IgA in the presence of stimulation (Figure 10), while the released IgA by iLN B cells were not detectable (data not shown). Interestingly, although not statistically significant, IgA levels are also increased in the basal condition, on the contrary we did not observe differences in the IgG levels.

Collectively, these data support our finding that the $Apc^{Min/+}$ mice are characterized by a generalized IgA skewing, leading us to conclude that the presence of the tumor determines a profound alteration and the activation of B cells.

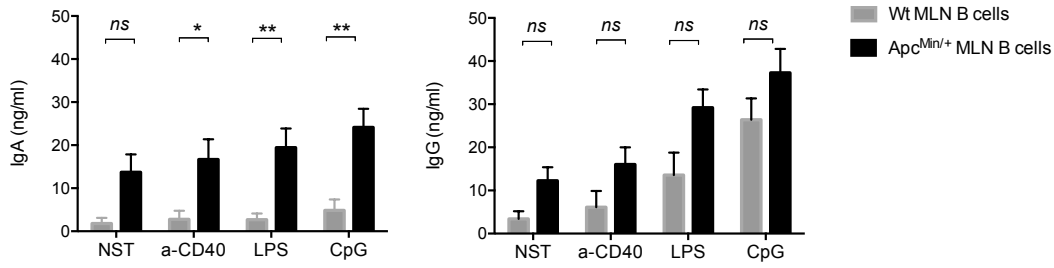


Figure 10. MLN B cells of the $Apc^{Min/+}$ mouse model produce more IgA than healthy controls. B cells purified from MLNs of WT or $Apc^{Min/+}$mice were cultured either alone (NST) or in the presence of a-CD40 mAb, LPS or CpG for 48 h. Cell supernatants were collected and IgA and IgG levels detected by ELISA.
*Mean + SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

I then decided to understand whether the IgA skewing is peculiar of only the $Apc^{Min/+}$ mouse or if it is common to other CRC models. Specifically, I focused our study on the colitis-associated CRC induced by AOM/DSS treatment and in mice subcutaneously injected with the MC38 CRC cell line. To this purpose, the capacity of B cells from different anatomical compartments to produce immunoglobulins and the antibody titers in sera were determined in healthy and CRC tumor-bearing mice.

Immunoglobulin concentrations in culture supernatants of untreated and *in vitro* stimulated B lymphocytes from spleen, MLNs and peritoneum, were evaluated in the AOM/DSS model (Figure 11, panel B). In spite of comparable release of IgG, following activation, the splenic B cells isolated from AOM/DSS tumor-bearing mice produce higher amounts of IgA compared to healthy B cells. Furthermore, increased amounts of IgA were released also by B cells purified from MLNs and peritoneal of AOM/DSS mice, compared to the healthy controls.

As shown in panel A of figure 11, when we quantify the levels of circulating immunoglobulins in the sera, we found that IgA, IgG, but not the IgM, resulted increased in AOM/DSS mice, compared to control mice.

The fact that in the AOM/DSS tumor-bearing mice, in addition to the increased IgA levels, also the IgG titers were increased compared to the healthy counterpart, highlights the induction of an overall change in the Ig balance due to the state of induced inflammation that contribute to the alteration of the physiologic conditions, not only in the mucosal response, but also in the systemic immune response.

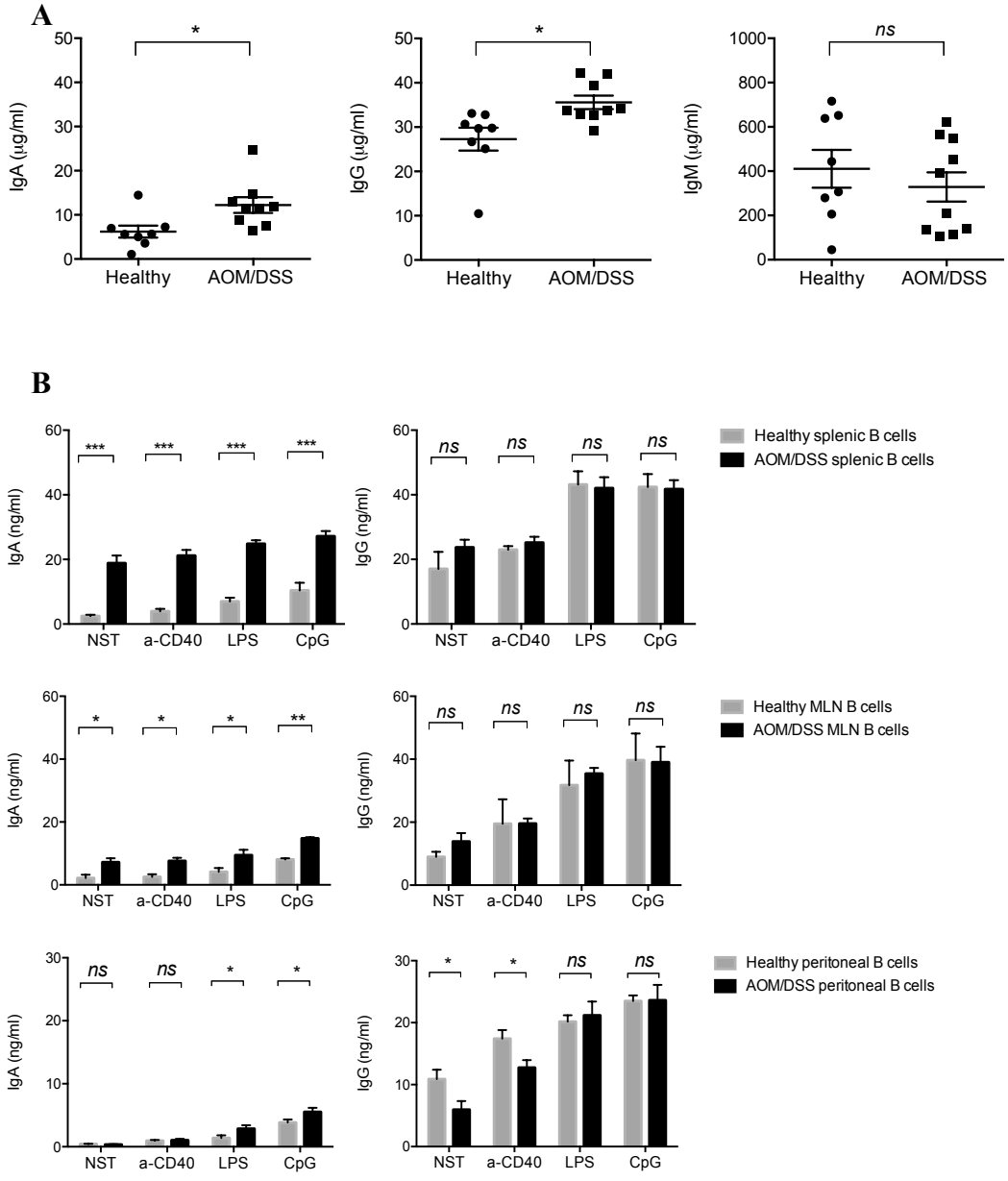


Figure 11. **Immunoglobulins levels in the AOM/DSS mouse model. A.** IgA, IgG and IgM levels in the AOM/DSS sera compared to the control mice were quantified by ELISA. **B.** B cells purified from spleen, MLNs and peritoneum of healthy or AOM/DSS tumor-bearing mice were cultured either alone (NST) or in the presence of a-CD40 mAb, LPS or CpG for 48 h. Cell supernatants were collected and IgA and IgG levels detected by ELISA.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

The same experiments were performed in the MC38 CRC mouse model. In this case, the levels of circulating IgA and IgG were not increased in tumor-bearing mice compared to the healthy counterpart (figure 12, panel A).

On the contrary, compared to healthy controls, splenic B cells from MC38 tumor-bearing mice are able to release higher levels of IgA (but not IgG or IgM), both in resting and stimulated conditions (figure 12, panel B).

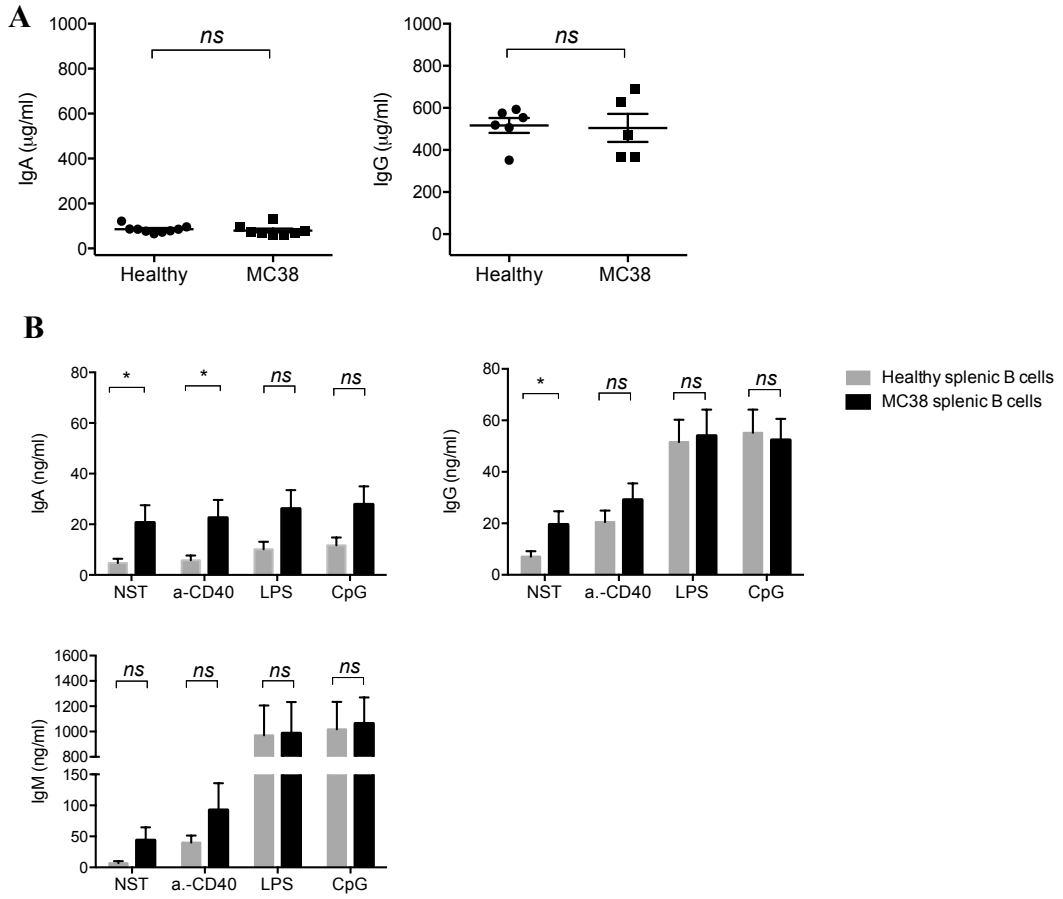


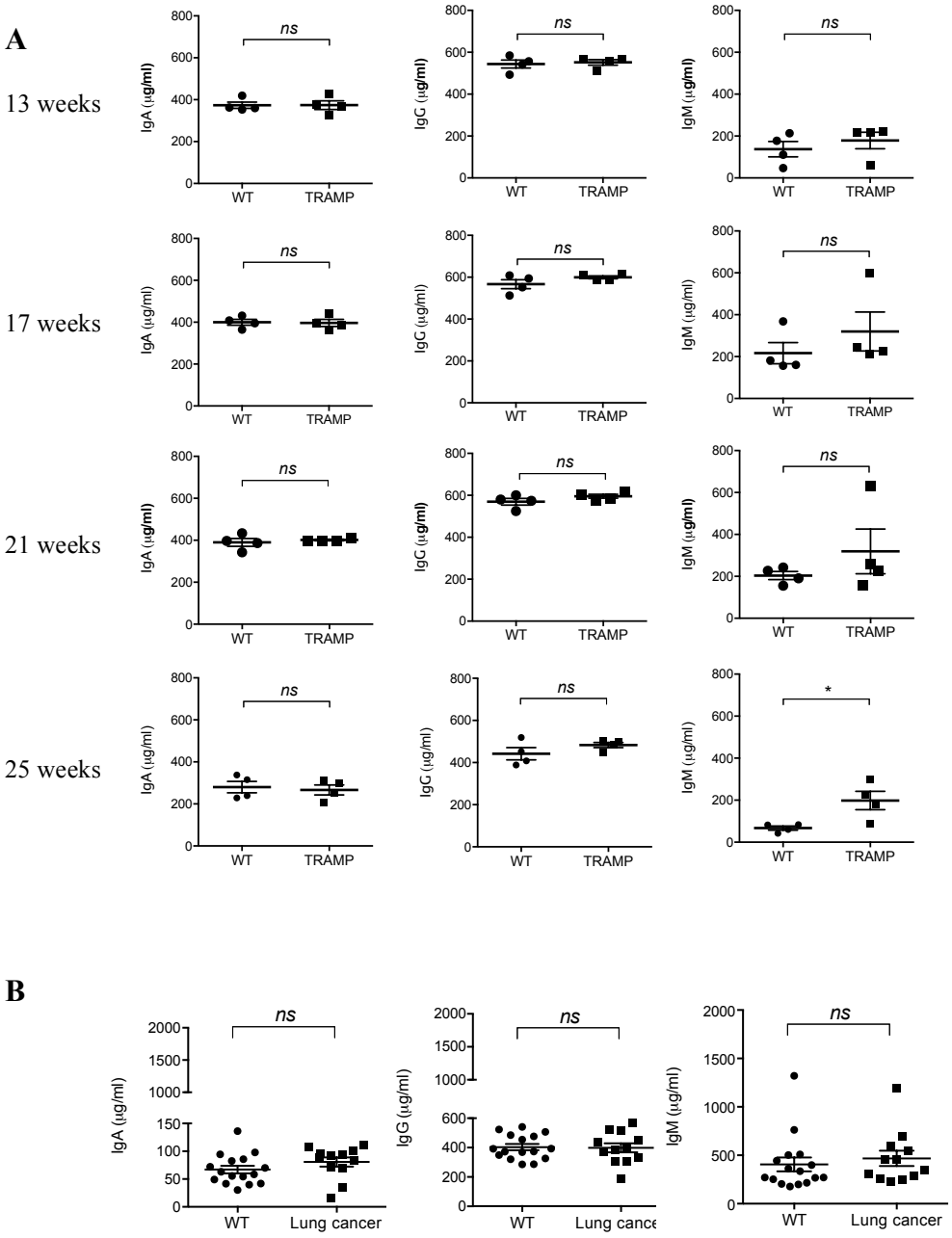
Figure 12. **IgA is not increased in sera of MC38 tumor-bearing mice, but splenic B cells have an increased capacity to produce IgA. A.** IgA and IgG levels in the sera of MC38 tumor-bearing mice compared to those of the healthy mice were quantified by ELISA. **B.** B cells purified from the spleen of healthy or MC38 tumor-bearing mice were cultured either alone (NST) or in the presence of a-CD40 mAb, LPS or CpG for 48 h. Cell supernatants were collected and IgA and IgG levels detected by ELISA.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

The totality of these data highlights that a general alteration of the Ig released by *in vitro* activated B lymphocytes from different compartments is peculiar of different CRC mouse models, except for the $Apc^{Min/+}$ mouse model. These results reflect the involvement of distant organs and the activation of the B cell immune response in several CRC mouse models during tumor progression. Therefore, I can conclude that the sole IgA skewing is peculiar of the $Apc^{Min/+}$ CRC tumor model, while in the other CRC bearing mice an overall change in the Ig balance is present compared to healthy mice.

3.1.4. IgA serum levels are not increased in murine tumor models unrelated to the intestinal tract

In order to understand if the IgA skewing is specifically associated only with the CRC $Apc^{Min/+}$ mice, I decided to detect the IgA production also in murine tumor models unrelated to the intestinal tract. Given the impossibility to take into analysis all the tumor types, I decided to detect the Ig levels in three representative models of solid tumor. I assessed the titers of circulating Ig in the TRAMP model (Shen and Abate-Shen, 2010), in mice orthotopically injected with the KP cell line as a model of lung cancer (Dimitrova et al., 2016), and in the breast cancer mouse model Delta16 HER2 (d16HER2) in which a splice variant of the human HER2 gene, called Delta16 HER2 (d16HER2) (Castagnoli et al., 2017), shows enhanced transforming activity and is associated with worse patient prognosis. All the tumor samples were analyzed and compared with their relative controls (Figure 13).

Panel A of figure 13 shows the Ig levels of TRAMP mice compared to WT mice. In order to assess eventual alterations during tumor progression, we measured the Ig levels in mice at different ages. At 13 weeks the mice have a normal epithelium; at 17 weeks the tumor is present at a low grade, differently from the high grade of the tumor present at 21 weeks. Finally, at 25 weeks mice present the adenocarcinoma. For each time point analyzed, no significant differences were found in the Ig levels, except for the IgM levels in 25 week-old TRAMP mice.


A
13 weeks
17 weeks
21 weeks
25 weeks
IgA (µg/ml)
IgG (µg/ml)
IgM (µg/ml)
ns
*
WT
TRAMP
B
Lung cancer

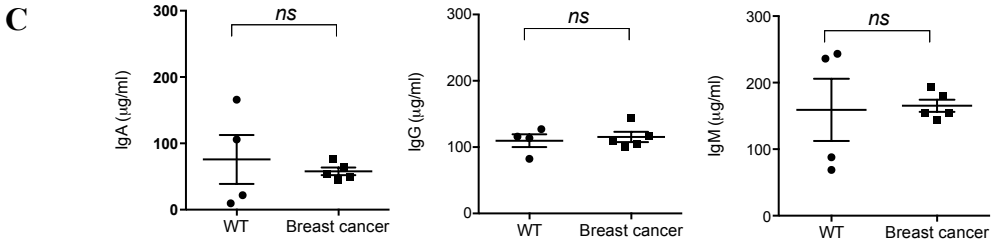


Figure 13. **IgA serum levels are not increased in mice with tumor not related to the intestinal tract.** Titers of circulating IgA, IgG and IgM in TRAMP mice **(A)**, lung cancer-bearing mice orthotopically injected with the KP cell line **(B),** and in the breast cancer mouse model Delta16 HER2 (d16HER2) **(C)** were detected by ELISA.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

The data obtained from the analysis of the mice orthotopically injected with the KP cell line and from the breast cancer d16HER2 mice are respectively reported in panel B and C of figure 13. Also in these cases, I could not appreciate any statistically significant difference in the Ig levels in the sera of the tumor-bearing mice compared to the sera of the control mice.

In conclusion, our experiments did not show differences in the Ig levels between intestinal unrelated tumor-bearing mice and their healthy controls. Therefore, I can confirm the specificity of the IgA skewing only in the $Apc^{Min/+}$ mice.

3.1.5. Tumor-bearing $Apc^{Min/+}$ mice have increased microbial translocation compared to WT mice

In our body the majority of activated B cells in the gut differentiate into IgA plasma cells. The mucosal surfaces are continuously exposed to potential harmful agents and IgA represent the first line of defence at these sites (Cerutti, 2008).

Tumor-bearing $Apc^{Min/+}$ mice have increased intestinal permeability compared to their WT counterpart (Puppa et al., 2011b). Since it is known that gut barrier dysfunction is linked to increased microbial translocation (Fink and Delude, 2005), I decided to assess whether the intestinal damage that triggers microbial translocation could be responsible of the observed IgA response. Therefore, I decided to dissect this mechanism as a possible response for the IgA increase in

the $Apc^{Min/+}$ and, to perform a comparative relation, I decided to investigate the same aspects in the AOM/DSS mice.

First of all, I decided to investigate if the increased IgA level is due to the alterations of the gut epithelial barrier that favour adenoma formation. Gut barrier dysfunction is linked to increased microbial translocation (Turner, 2009) and, in this view, the IgA skewing might be part of the systemic inflammatory response to the entry of bacterial cell components or intact bacteria in circulation.

The damage of the gut barrier allows microbial products and viable bacteria to translocate from the intestinal lumen to extra-intestinal organs. The majority of the venous blood from the intestinal tract is drained into the portal circulation, which is part of the dual hepatic blood supply. The liver is, therefore, the first organ in the body to encounter gut-derived bacteria (Wang et al., 2015). For this reason, I decided to detect the bacterial growth in the liver of $Apc^{Min/+}$ and AOM/DSS mice in order to assess the presence of a higher bacterial level in the tumor-bearing mice in comparison with the bacterial levels in the livers of the control mice. Although the obtained data are not statistically significant, in livers of $Apc^{Min/+}$ mice a positive trend was found, both for aerobic and anaerobic bacteria, with about one log-difference in the number of colonies forming units (CFU) compared to the WT livers. An opposite trend was found, instead, in the livers of AOM/DSS mice in which I did not find increased bacterial growth compared to the healthy controls (Figure 14).

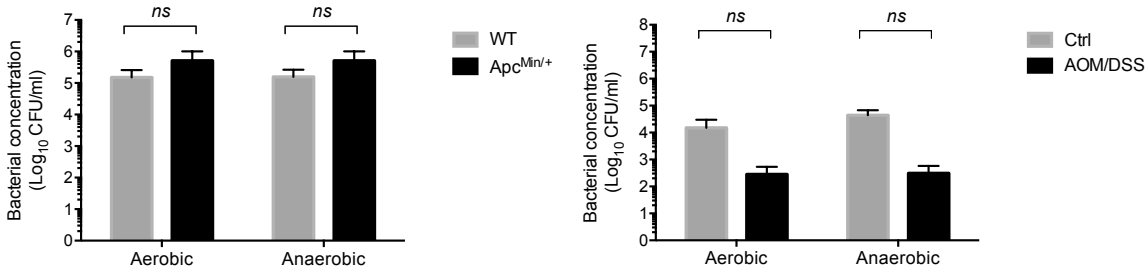


Figure 14. **Livers of $Apc^{Min/+}$ mice have increased levels of bacteria compared to the WT counterpart.** Aerobic and anaerobic bacteria levels in $Apc^{Min/+}$ and AOM/DSS mice compared to the WT controls were detected in the mouse homogenised livers. The whole liver was homogenized in sterile PBS. Serial dilutions were plated in LB, TSA, Orientation, Brucella and Chocolate media. The plates were incubated at 37 °C under 5% CO_2 pressure or in anaerobic condition for 36 hours and colony-forming units (CFU). were enumerated.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

The contribution of bacterial species to CRC development is increasingly acknowledged and several studies found that specific bacterial groups were common in colorectal cancer cases than control specimens (Dahmus et al., 2018; Sun and Kato, 2016). Therefore, in this work I also assessed the diversity of the bacterial genus identified in CRC samples compared to the healthy controls (Figure 15).

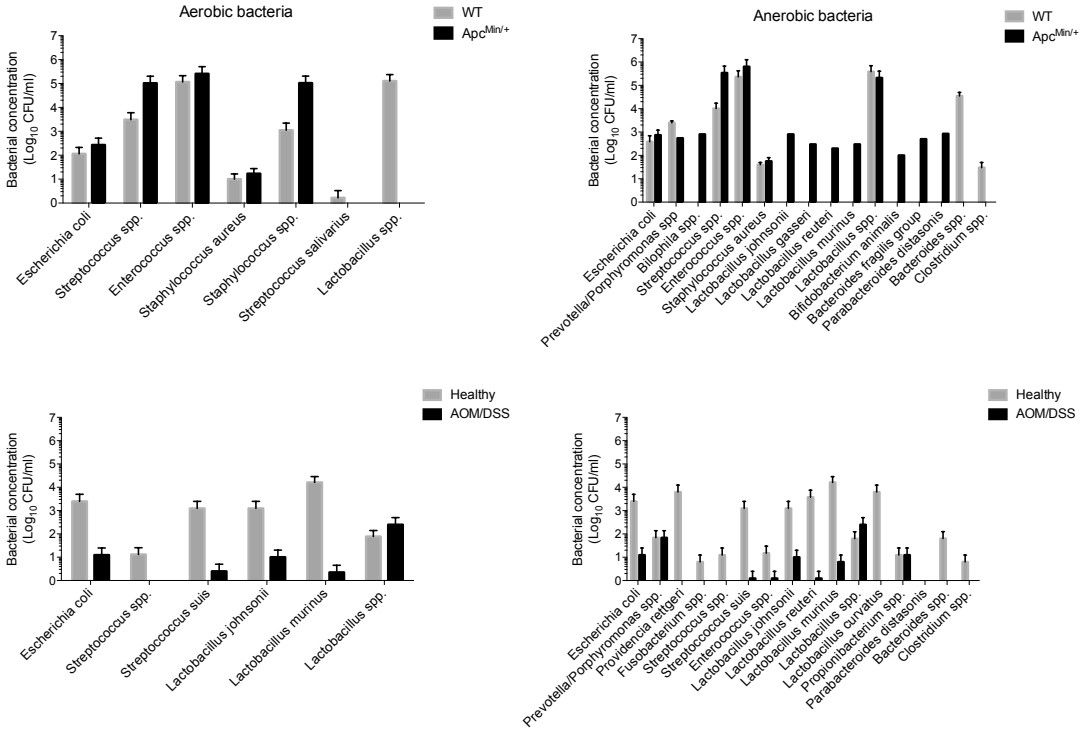


Figure 15. **Aerobic and anaerobic bacterial genus from Apc$^{Min/+}$ and AOM/DSS livers.** Quantification of bacterial genus identified in the Apc$^{Min/+}$ and AOM/DSS livers compared to the healthy livers was obtained after the enumeration of CFU/ml growth in the liver cultures.

Contrarily to the AOM/DSS mouse model in which I did not observe increased bacterial growth compared to the healthy cultures, I found a higher number of bacterial genus in the Apc$^{Min/+}$ livers compared to the WT, especially for the anaerobic species. In particular, I found an increased concentration of *Streptococcus* and the growth of *Bilophila, Bifidobacterium* and *Bacteroides*

fragilis that were instead absent in the WT mice.

These results, lead us to hypothesize that the intestinal damage could first allow the translocation of the aerobic bacteria in the near tissues that consume the oxygen. This process could prepare the appropriate niche for the anaerobic bacteria that, in different environment do not translocate and are not able to persist. The presence of anaerobic species lead us also to suppose that the bacterial translocation is a persistent phenomenon in the $Apc^{Min/+}$ mouse model, otherwise I could not observe the presence of anaerobic bacterial species that request the preparation of the environment by the aerobic species.

3.1.6. Tumor-bearing $Apc^{Min/+}$ mice have increased 16S prokaryotic rRNA compared to WT mice

Although a large number of intestinal bacteria cannot be cultured, their identification has become possible by using DNA genome sequencing

To confirm the obtained data in the $Apc^{Min/+}$ mouse model, whole blood samples from $Apc^{Min/+}$ and WT mice were subjected to the DNA purification. I then performed the 16S sequencing in order to assess the relative abundance of each bacterial genus.

The sequencing data confirmed the presence of a higher bacterial content in the $Apc^{Min/+}$ samples compared to the WT, in fact the number of the obtained reads is about twice (Figure 16).

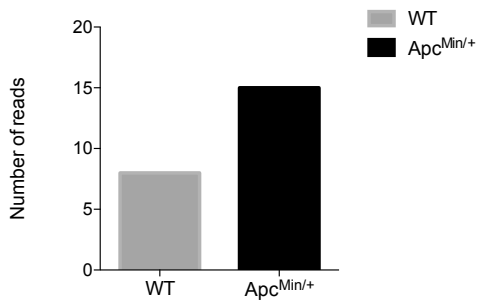
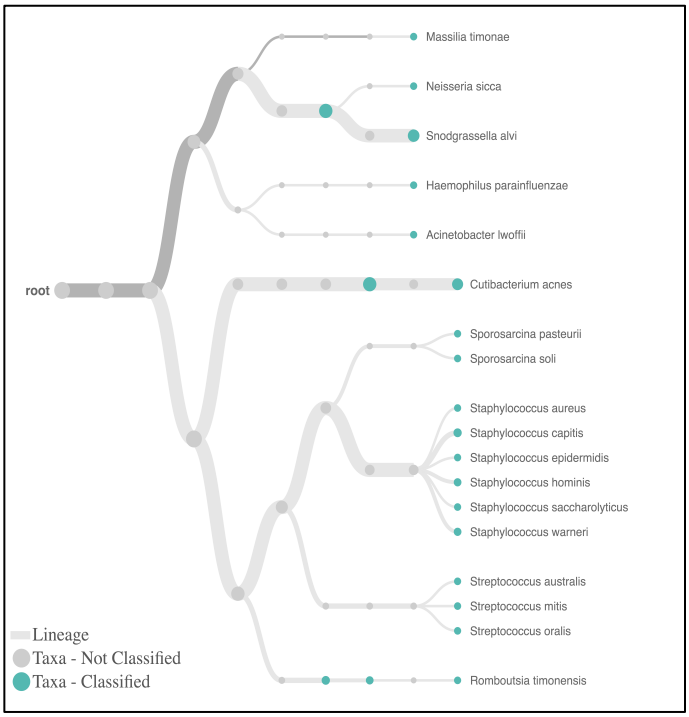


Figure 16. 16S content in the WT and $Apc^{Min/+}$ DNA. Number of obtained reads through DNA sequencing in the $Apc^{Min/+}$ DNA compared to the WT DNA.

The sequencing results have also furnished us information about the totality of bacterial species identified in the $Apc^{Min/+}$ and in the WT groups (Figure 17, panel A). The analysis of the bacterial species identified, underlie the presence of a vast panel of the bacteria in the $Apc^{Min/+}$ samples compared to those present in the WT mice. In particular, *Cutibacterium acnes*, *Snodgrassella alvi* and *Staphylococcus capitis* resulted the bacteria most represented in the $Apc^{Min/+}$ mice (Figure 17, panel B). In detail, the bacterial species classified in the WT mice represent the 25% of the totality of the species identified, while the species in the $Apc^{Min/+}$ samples represented the 60% of the identified bacterial taxa. The 12% of the taxa were shared between the two groups (Figure 17, panel C).

A


root
Massilia timonae
Neisseria sicca
Snodgrassella alvi
Haemophilus parainfluenzae
Acinetobacter lwoffii
Cutibacterium acnes
Sporosarcina pasteurii
Sporosarcina soli
Staphylococcus aureus
Staphylococcus capitis
Staphylococcus epidermidis
Staphylococcus hominis
Staphylococcus saccharolyticus
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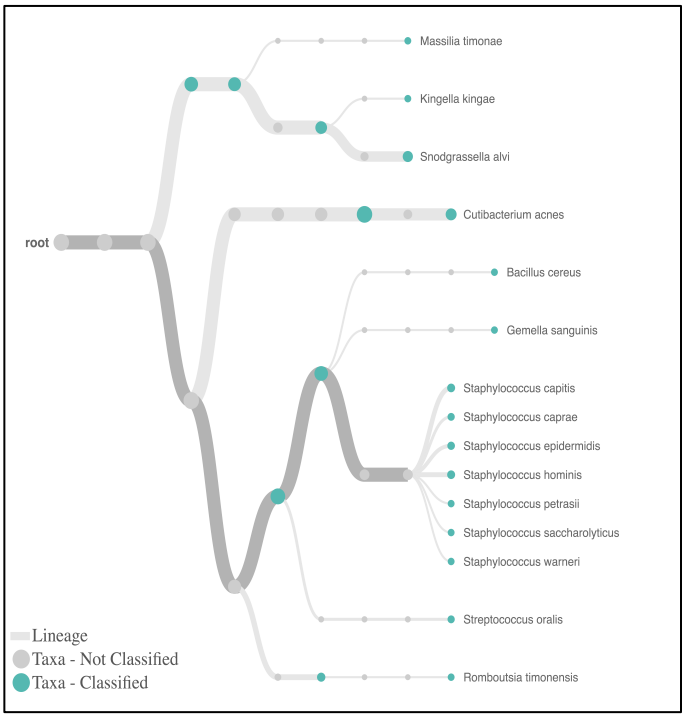
Streptococcus mitis
Streptococcus oralis
Romboutsia timonensis
Lineage
Taxa - Not Classified
Taxa - Classified


root
Massilia timonae
Kingella kingae
Snodgrassella alvi
Cutibacterium acnes
Bacillus cereus
Gemella sanguinis
Staphylococcus capitis
Staphylococcus caprae
Staphylococcus epidermidis
Staphylococcus hominis
Staphylococcus petrasii
Staphylococcus saccharolyticus
Staphylococcus warneri
Streptococcus oralis
Romboutsia timonensis
Lineage
Taxa - Not Classified
Taxa - Classified

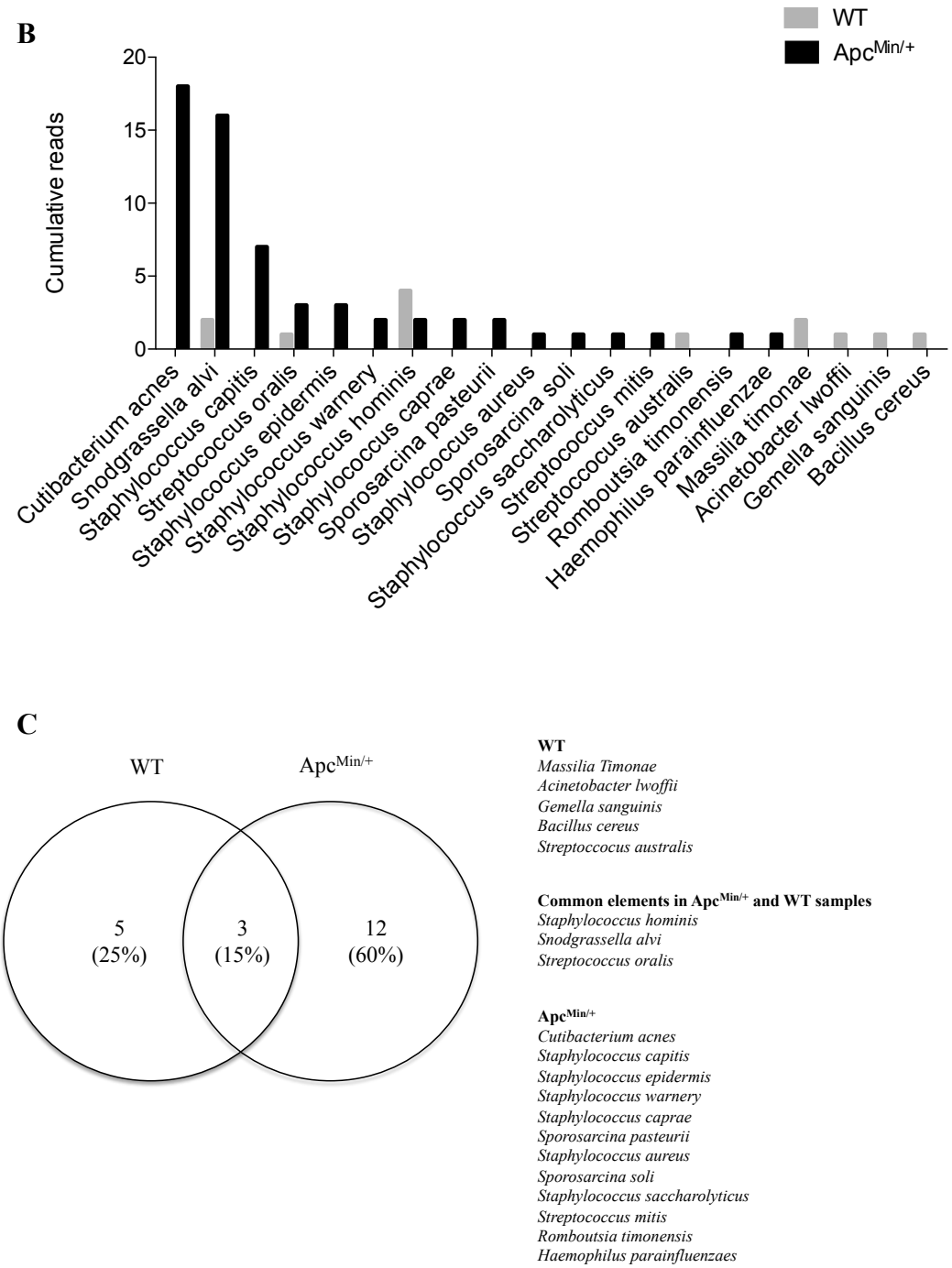


Figure 17. 16S metagenomic analysis. A. Totality of bacterial species identified in the $Apc^{Min/+}$ and in the WT groups. The images are relative to each of the two run performed. **B.** Bacterial species detected in the $Apc^{Min/+}$ compared to those in the WT mice. **C.** % of bacterial species found in the WT and in the $Apc^{Min/+}$ mice.

The collected data indicate that a marked microbial translocation occurs specifically in the $Apc^{Min/+}$ mouse model. This microbial translocation could also explain the increased IgA levels found at a systemic level and in the different compartments analysed. Therefore, I conclude hypothesizing that the microbial translocation could be a possible mechanism responsible for IgA increase in the $Apc^{Min/+}$ mice.

To further validate our hypothesis about the presence of a positive correlation between IgA skewing and increased intestinal permeability, I am now planning to measure the titers of circulating Abs in $Apc^{Min/+}$ and $Apc^{Min/+}$-$JAM^{-/-}$ mice, under basal and inflammatory conditions. $JAM^{-/-}$ mice present increased mucosal permeability and a major susceptibility to DSS-induced colitis, caused by the absence of the JAM-A protein expressed at tight junctions of epithelial and endothelial cells (Laukoetter et al., 2007).

3.2. MDSCs can induce an aggressive phenotype of B cells in CRC

3.2.1. The splenic microenvironment is altered in CRC

Mion and colleagues have recently demonstrated the accumulation of MDSCs in the $Apc^{Min/+}$ mice compared to the WT controls and associated the IgA release by B cells with the presence of MDSCs. They demonstrated, in fact, that B cells in co-culture with MDSCs released a significantly higher amount of soluble IgA (Mion et al., 2017).

On the basis of literature data about the accumulation of MDSCs in the tumor context (Ostrand-Rosenberg and Sinha, 2009), I decided to assess if also in the CRC mouse models studied in this work, I could observe the MDSCs accumulation.

Metastasis is the cause of most of tumor-related deaths, I therefore decided to analyse also the model of CRC metastasis to the liver in which the tumor is induced by cecum implantation of the highly aggressive CT26-FL3 cell line (Zhang et al., 2013).

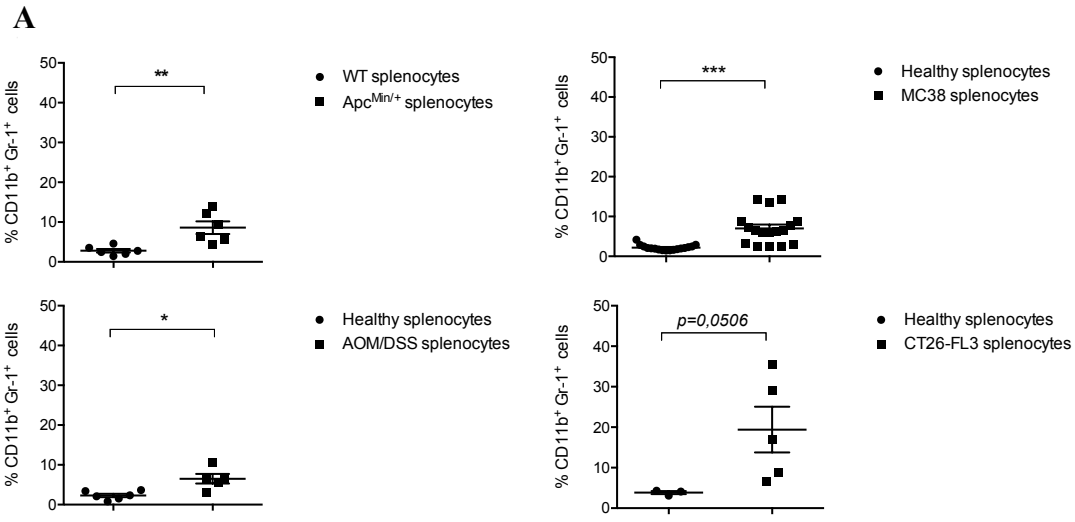
The figure 17 shows an expanded amount of $CD11b^{+}Gr1^{+}$ immature myeloid cells in the spleen of $Apc^{Min/+}$, MC38, AOM/DSS and CT26-FL3 tumor-bearing mice compared to the healthy controls (Figure 18, panel A). I also analyzed the

distribution of the two main subsets of MDSCs in the $CD11b^+$ population of myeloid cells: PMN-MDSCs and M-MDSCs. I found a predominance of PMN-MDSCs in $Apc^{Min/+}$, CT26-FL3 and in AOM/DSS mouse models, while in the MC38 tumor-bearing mice the M-MDSCs subset is more expanded (Figure 18, panel B).

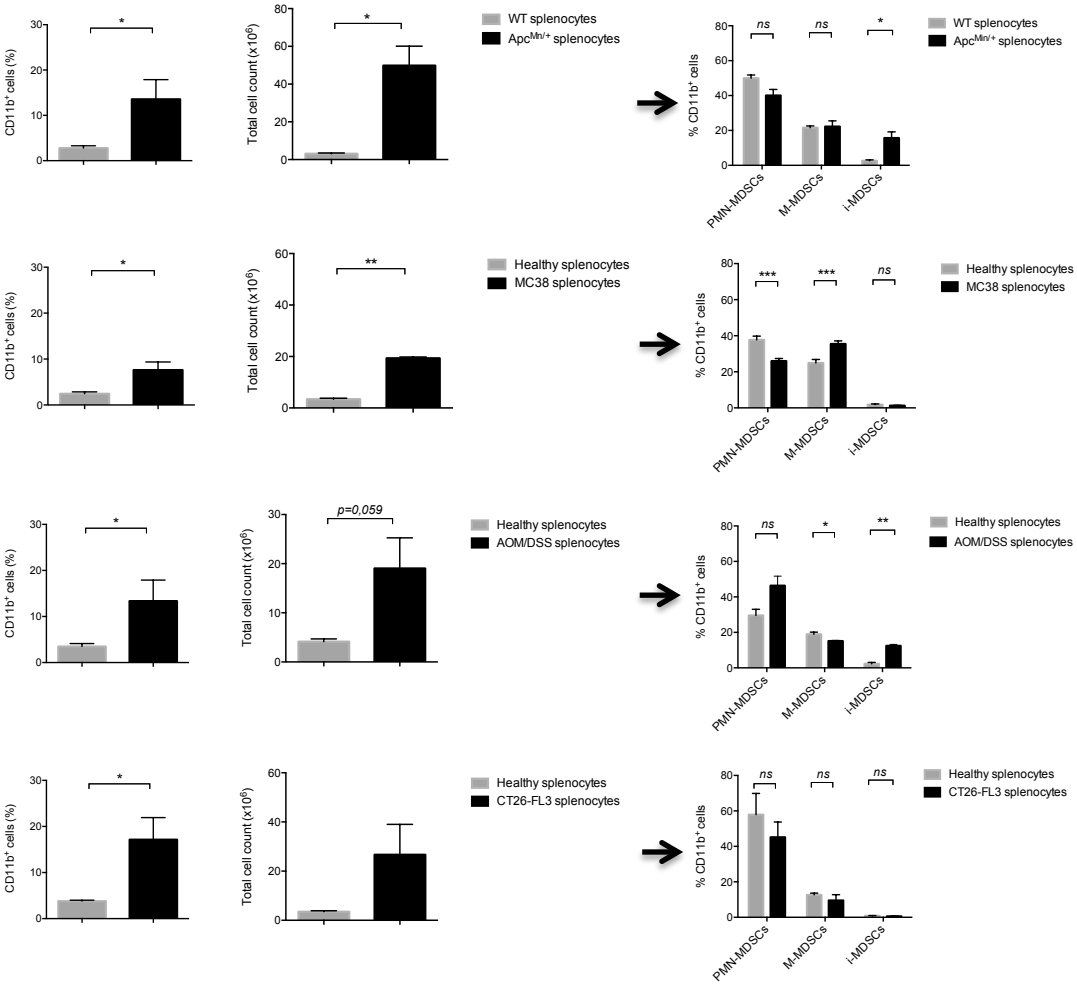
Furthermore, only in the $Apc^{Min/+}$ and in the AOM/DSS mice I found the presence of a third population of immature myeloid cells, the iMDSCs, which presents intermediate levels of the markers Ly6C and Ly6G (figure 18, panel B).

In the MC38 tumor-bearing mice, the amount of $CD11b^+Gr1^+$ cells was analyzed also in the tumor mass and I found a significant accumulation of MDSCs in the tumor MC38 compared to the healthy and the tumor spleens (figure 18, panel C).

Furthermore, when I analysed the distribution of the MDSC subsets in the $CD11b^+$ cells of the MC38 tumor mass, I found a prevalence of the PMN-MDSC subset, contrarily to what observed in the spleen of MC38 tumor-bearing mice (figure 18, panel C).



B


CD11b+ cells (%)
Total cell count (x10^6)
WT splenocytes
Apc^Min/+ splenocytes
% CD11b+ cells
PMN-MDSCs
M-MDSCs
i-MDSCs
Healthy splenocytes
MC38 splenocytes
AOM/DSS splenocytes
CT26-FL3 splenocytes
p=0,059
ns

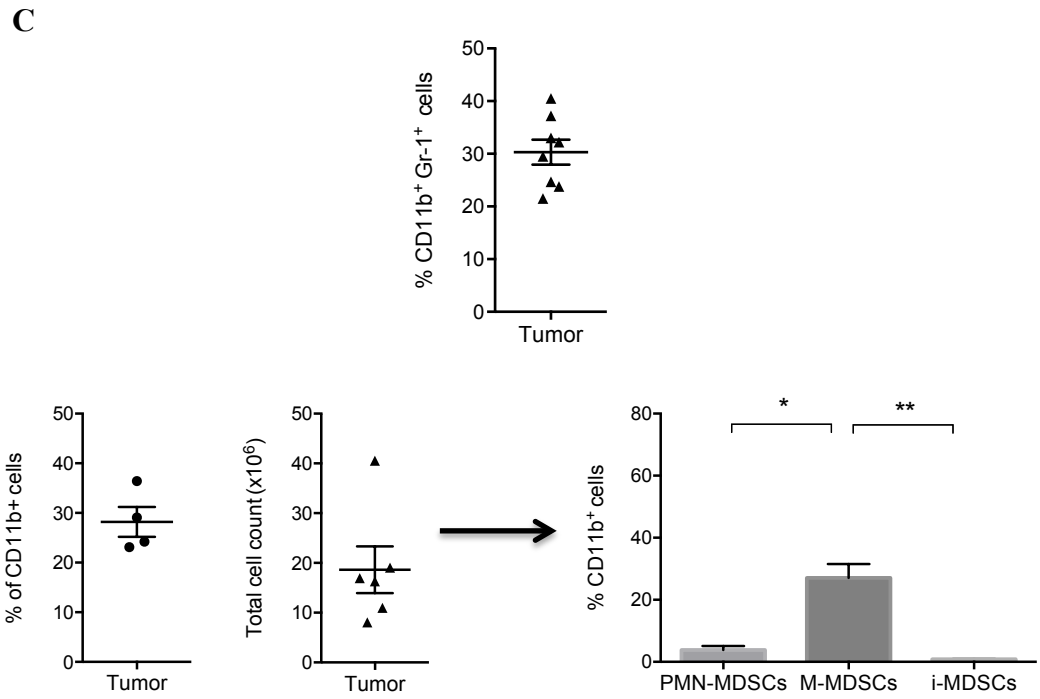


Figure 18. The myeloid composition of CRC-bearing mice compared to healthy mice. A. Flow cytometry analyses of the total MDSCs ($CD11b^+Gr1^+$) were performed on splenocytes of MC38, AOM/DSS, CT26-FL3 tumor-bearing and $Apc^{Min/+}$mice compared to the healthy controls. **B.** Frequencies of the specific subsets of MDSCs. Analyses of PMN-MDSC, M-MDSC and i-MDSC subsets in tumor-bearing mice compared to the healthy controls were performed by flow cytometry. **C.** Flow cytometry analyses of total MDSC distribution and frequencies of the specific subsets in the MC38 tumor site.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant*

3.2.2. MDSCs isolated from MC38 tumor-bearing mice and $Apc^{Min/+}$ mice have suppressive activity in presence of LPS

The phenotypical characterization of MDSCs is not sufficient to define the immature myeloid population as "suppressive". The ability of MDSCs to suppress the B cell proliferation was measured in terms of proliferation. B cells, purified from healthy spleens, and the MDSC subsets, purified from the spleens of tumor-bearing mice, were co-cultured and the suppressive power of MDSCs in inhibiting the B cell proliferation was quantified. The same experiments were performed in the $Apc^{Min/+}$ and in MC38 models. The obtained data showed that B cell proliferation is suppressed in presence of PMN-MDSCs and M-MDSCs purified

from MC38 tumor-bearing mice, after stimulation with LPS. A similar trend was obtained in presence of PMN-MDSCs and M-MDSCs purified from the spleens of $Apc^{Min/+}$ mice (Figure 19).

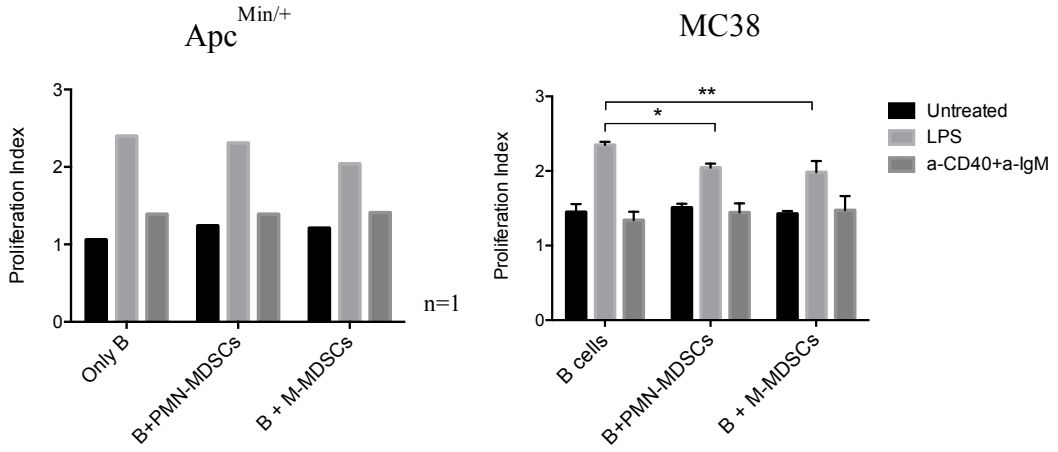


Figure 19. Proliferation assay in the B/MDSC co-cultures. PMN-MDSCs or M-MDSCs purified from the spleens of $Apc^{Min/+}$ mice or MC38 tumor-bearing mice e were co-cultured (1:1 ratio) either alone (Untreated) or in the presence of LPS or a-CD40 mAb + a-IgM for 72h. Cells were recovered, stained for CD19 and analyzed by flow cytometry.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

3.2.3. Characterization of MC38 TIL-B

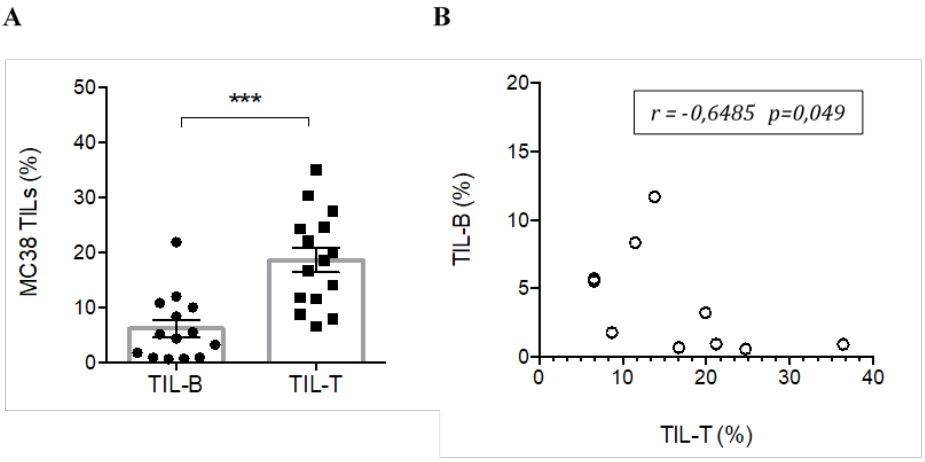
The progress in the study of the immune microenvironment have mainly highlighted the role of T cells in the anti-tumor immune response (Gu-Trantien et al., 2013; Hung et al., 1998); while the role of B cells in modulating the immune response to tumors is less well understood.

B cells are the second most abundant tumor-infiltrating lymphocyte and they can have a dual role. First, they can inhibit tumor development through the production of tumor-reactive antibodies, promoting tumor killing by NK cells, phagocytosis by macrophages, and the priming of $CD4^+$ and $CD8^+$ T cells. On the other hand, B cells can promote tumor development through the production of autoantibodies and tumor growth factors (Yuen et al., 2016). Furthermore, the subset of

regulatory B cells can, directly and indirectly, suppress Th1 and $CD8^+$ cytolytic T cell responses (Rosser and Mauri, 2015).

In this scenario, the target of specific B cell subsets may be of therapeutic value in cancer; therefore I decided to investigate the presence and the characteristics of TIL-B in the MC38 tumor.

First of all, I assessed if TIL-B were present in the MC38 tumor mass (Figure 20, panel A). In order to understand if the presence and the amount of TIL-B depend on the tumor size and weight, I performed a correlation analysis, but I did not find any correlation (data not shown). On the contrary, a negative correlation between the % of TIL-B and TIL-T was found. This evidence lead us to suppose the presence of a finely regulated equilibrium mechanism in the recruiting of the immune cells in the tumor site (Figure 20, panel B). The same correlation was performed for the splenic $CD19^+$ B cells and the $CD4^+$ T cells in the MC38 spleen compared to the healthy spleen, but I did not find differences between the healthy and the tumor conditions (data not shown).



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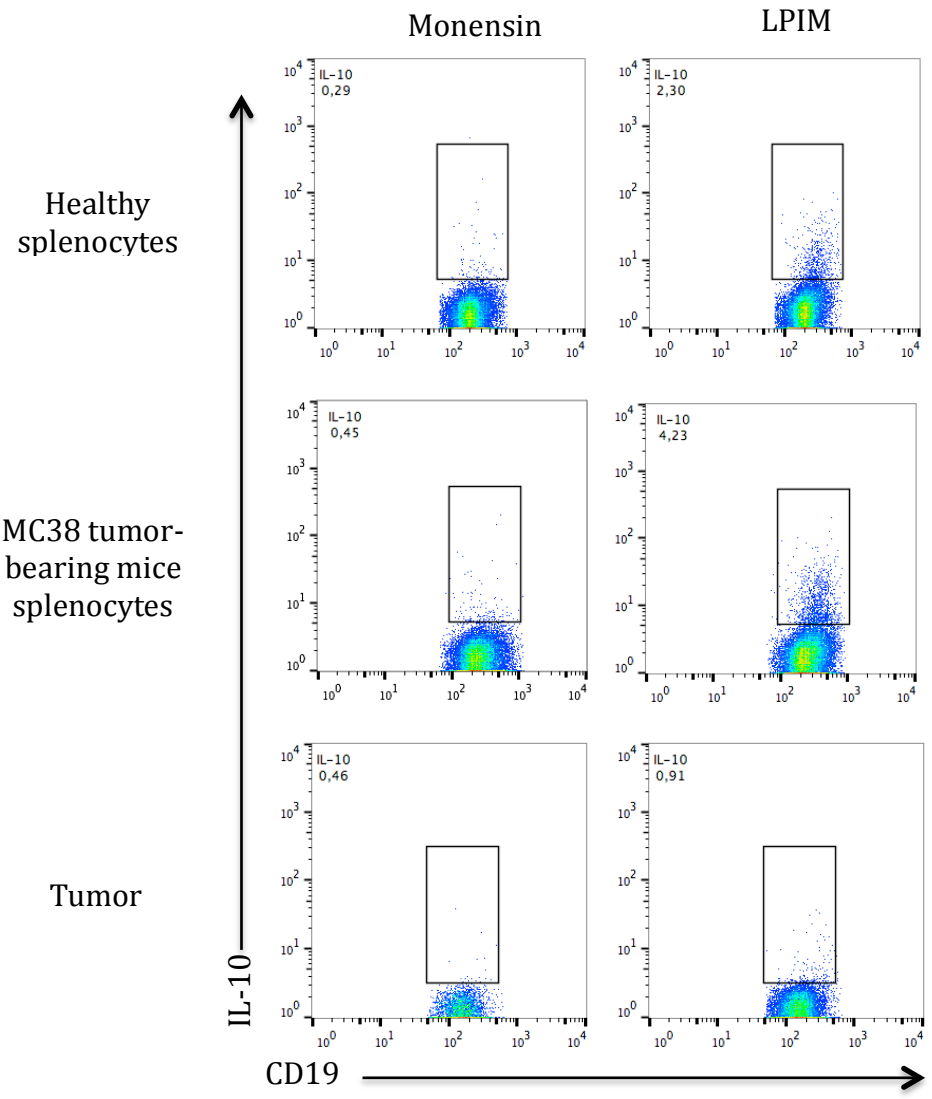
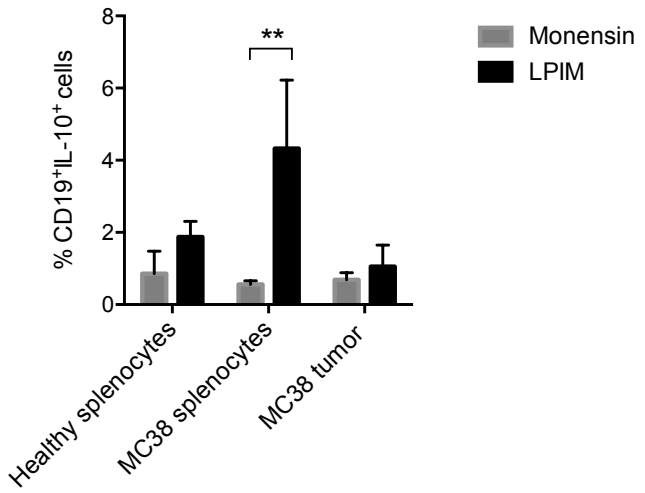

Monensin
LPIM
Healthy splenocytes
MC38 tumor-bearing mice splenocytes
Tumor
IL-10 0,29
IL-10 2,30
IL-10 0,45
IL-10 4,23
IL-10 0,46
IL-10 0,91
IL-10
CD19


% CD19+IL-10+ cells
**
Monensin
LPIM
Healthy splenocytes
MC38 splenocytes
MC38 tumor

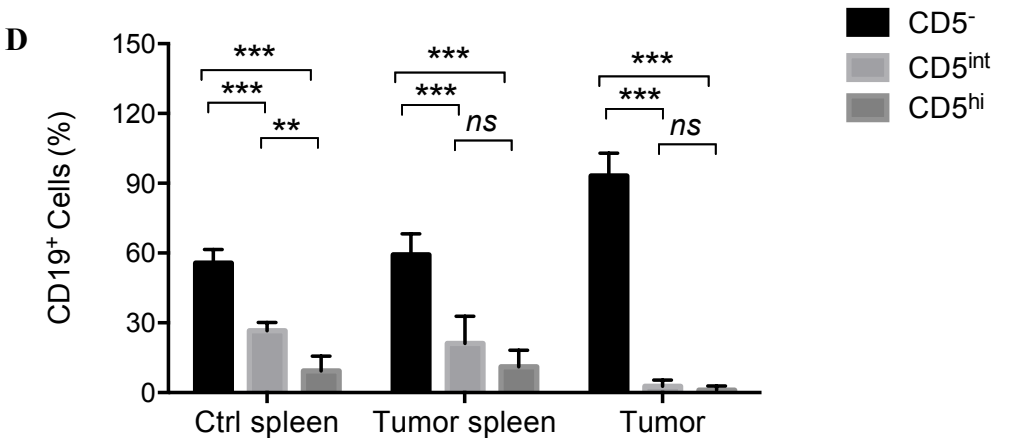


Figure 20. TIL-B/TIL-T distribution in the MC38 tumor. A. The percentage of MC38 $CD4^+$ TIL-T and $CD19^+$ TIL-B was assessed by flow cytometry analyses. **B.** The Spearman test was used for the correlation analyses between the percentage of TIL-T and TIL-B in the MC38 tumor. **C**. The frequency of B IL-10 competent B cells following a 5 h stimulation with Monensin or LPS, PMA, ionomycin and monensin (LPIM) was analyzed in freshly isolated splenocytes or tumor cells. The frequencies of IL-10^+ cells among total $CD19^+$ lymphocytes are reported. **D**. The TIL-B phenotype was assessed by flow cytometry analyses on freshly digested splenic and tumor cells analyzing the CD5 expression on $CD19^+$ cells.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*
*Test di Spearman (*** r=1).*

I next decided to investigate whether the TIL-B have regulatory features. The main mechanisms of immune suppression involves the IL-10 production and release (Rosser and Mauri, 2015). Therefore, I decided to analyze the expansion of the IL-10 competent B cells population. As reported in the figure 20 (panel C), I observed an expansion of IL-10^+ B cells in the spleen of MC38 tumor-bearing mice compared to the healthy controls, but, although a small population of $CD19^+$ TIL-B was detected compared to the $CD19^+$ population in the spleens, I found a small percentage of $CD19^+$ IL-10^+ B cells in the tumor mass. These data are in accordance with several works in the literature that describe TIL-B as positive regulators of the anti-tumor immune response (Lebien and Tedder, 2008).

Finally, I performed an additional flow cytometry analysis on splenic B cells and TIL-B using the CD5 marker, that allows distinguishing the two main populations of B1 cells: the $CD5^+$ B1-a and the $CD5^-$ B1-b cells. As shown in the figure 20,

(panel D), I found that the $CD19^+$ B cells in the MC38 tumor-bearing spleens and in the healthy spleens were mainly $CD5^-$, while a lower percentage of $CD5^{int}$ and $CD5^+$ populations were present. In the tumor, instead, I found that the totality of TIL-B was negative for the CD5 marker, infact the $CD5^{int}$ and $CD5^+$ populations were almost absent. This data allows us to only exclude that the TIL-B are B1-a cells.

3.2.4. PMN-MDSCs promote the expansion of IL-10 competent B cells in the spleen of $Apc^{Min/+}$, MC38 and AOM/DSS tumor models, but not the release of IL-10

The reported alteration of the splenic CRC microenvironment in terms of MDSCs accumulation, compared to the physiologic state, lead us to investigate the crosstalk between MDSCs and B cells in the tumor setting. Evidences of the interaction between B cells and MDSCs have been already reported, but further investigations are needed (Xu et al., 2017). Therefore, I decided to first unveil the influence of MDSCs in the field of IL-10 competent B cells.

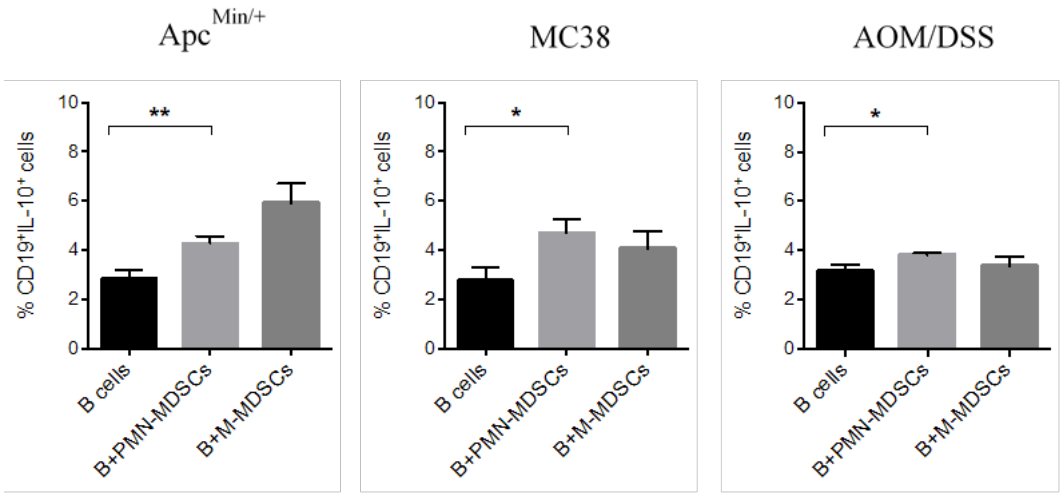


Figure 21. IL-10 competent B cells are expanded after co-culture with PMN-MDSCs. IL-10 competent B cell expansion was evaluated by flow cytometry analyses after 48h of co-culture between purified splenic B cells and MDSCs. The intracellular staining for IL-10 was performed following 5 h stimulation with LPS, PMA, ionomycin and monensin (LPIM).
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

To this purpose, I isolated PMN- and M-MDSCs from the spleen of $Apc^{Min/+}$, MC38 tumor-bearing mice and AOM/DSS treated mice and co-cultured them with healthy splenic B cells. I then evaluated by flow cytometry analyses the ability of MDSCs to induce the expansion of IL-10 competent B cells (Figure 21). PMN-MDSCs isolated from all the CRC models are able to induce the expansion of IL-10 competent B population. In addition, M-MDSCs isolated from $Apc^{Min/+}$ mice, showed a positive trend of induction of IL-10 competent B cells, although it is not statistically significative.

The expansion of IL-10–competent B cells and the production of IL-10 are two differentially regulated and distinct processes (Mion et al., 2014). Therefore, I also measured the released IL-10 in the supernatants of the co-cultures between splenic B cells and the PMN- and M-MDSCs (Figure 22).

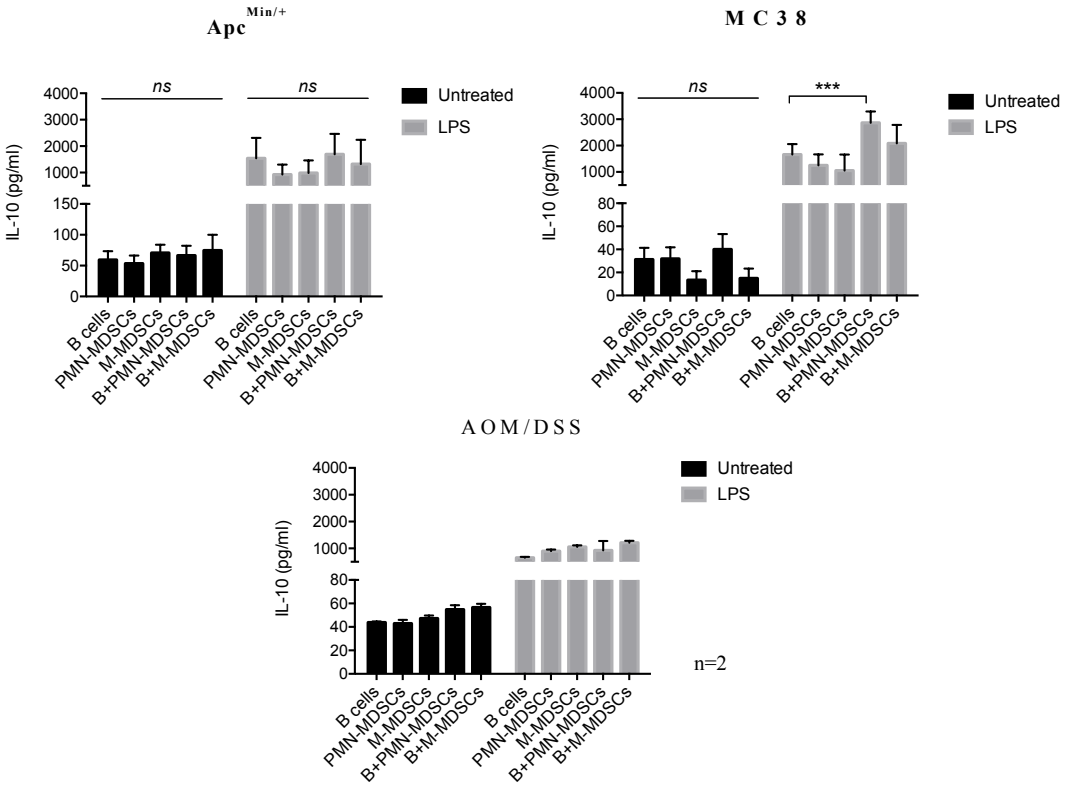


Figure 22. IL-10 release by B cells after co-culture with MDSCs. IL-10 secretion after 48h of B/MDSCs co-culture. The cells were cultured either alone (Untreated) or in the presence of LPS (positive control). Cell supernatants were collected and IL-10 levels detected by ELISA.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant*

As shown in figure 22, I did not find an increased release of IL-10 in the co-culture, compared to the B cells alone in the resting condition. The LPS stimulus has been used as positive control and, also in this case, I did not find any variation in the IL-10 release between the co-cultures and the B cells alone. The only difference was found in the co-culture B+PMN-MDSCs compared to the only B condition in the MC38 model that could be due to an additive effect in the IL-10 released by both B cells and the PMN-MDSCs. These data lead us to suppose that MDSCs do not induce the release of IL-10, but could prepare and instruct the B cells to become competent for a suppressive function when an appropriate and context-specific second signal will be encountered.

3.2.5. MDSCs induce the expression of IgA, PD-L1 and FasL on B cells in MC38 CRC

In addition to the mechanism driven from the anti-inflammatory cytokine IL-10, many pieces of evidence reported the presence of a B cells subset presenting killer functions due to the expression of ligands that induce death in target cells, such as PD-L1 and FasL (Green, et al., 2003). Therefore, I decided to define the phenotype and the function of B cells in the CRC context. First of all, I investigated the expression of FasL and PD-L1 on B cells, in both the tumor-derived and the healthy splenocytes. The analysis was also performed in the MC38 tumor mass (Figure 23).

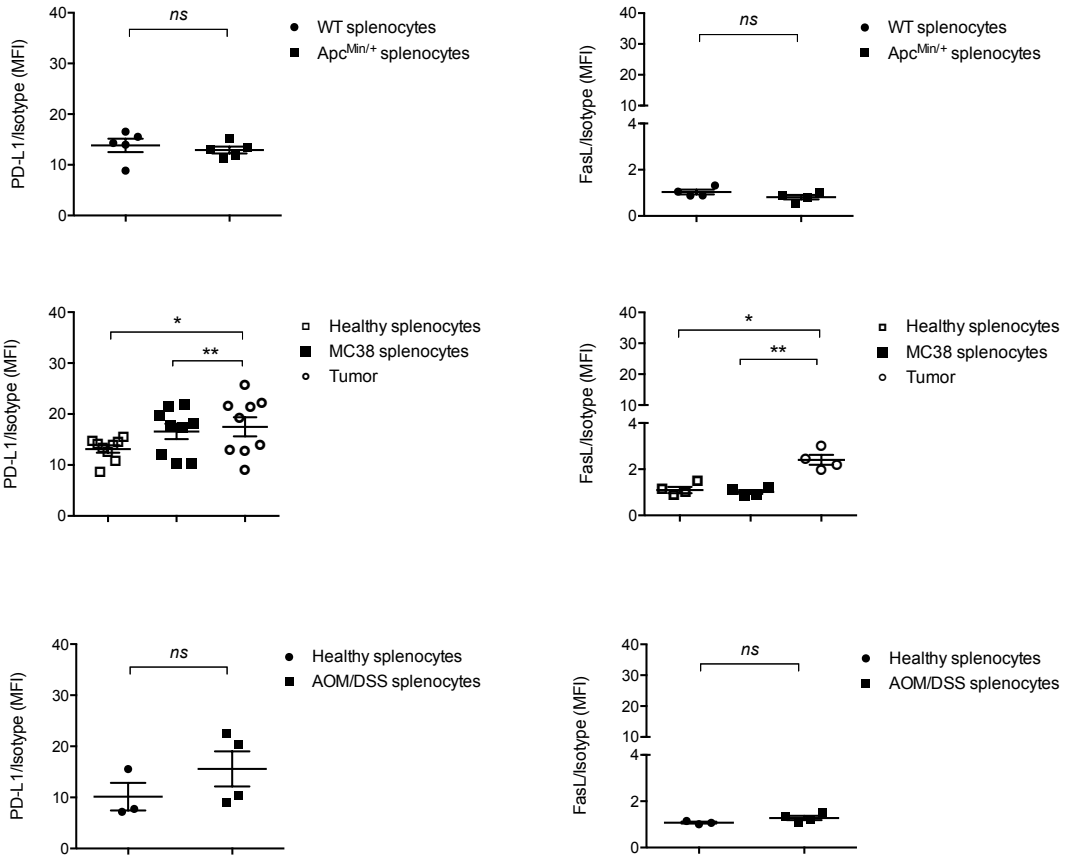


Figure 23. PD-L1 and FasL expression in splenic Apc$^{Min/+}$, MC38, AOM/DSS and healthy CD19^{+} B cells. PD-L1 and FasL expression was evaluated by flow cytometry on splenic CD19^{+} cells from tumor-bearing mice compared to the healthy splenocytes after spleen digestion. In the MC38 tumor-bearing mice the same analysis was performed on tumor infiltrating CD19^{+} B cells.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

As shown in the figure 23, no differences were detected in the B cells isolated from tumor-bearing mice compared to the healthy B cells in the spleens of Apc$^{Min/+}$, MC38 and AOM/DSS tumor models. On the contrary, I found a statistically significant upregulation of the PD-L1 and FasL markers in the MC38 TIL-B compared to both, the healthy and the tumor spleens. This data lead us to hypothesize that the tumor environment could educate TIL-B to become "aggressive" in order to suppress the anti-tumor immune response and to favour the tumor progression.

Given the ability of MC38 MDSCs to induce the expansion of IL-10 competent B

cells, I hypothesize that they could be responsible for the education of B cells in the tumor context. Therefore, I performed *in vitro* experiments to deepen the B/MDSC crosstalk in the MC38 tumor-bearing mice compared to the healthy mice.

MDSCs and B cells were co-cultured (1:1 ratio) and, after 48 hours, the expression of PD-L1 and FasL was analyzed. As shown in figure 24 (panel A), I found a significant up-regulation of PD-L1 and FasL in the B/MDSCs co-culture in presence of LPS, as compared to B cells alone. In particular, I found that PD-L1 was upregulated in presence of both the PMN-MDSC and the M-MDSC subsets, while the expression of FasL was significantly increased only in presence of PMN-MDSCs. The same experiments were performed with MDSCs isolated from $Apc^{Min/+}$ mice, but we did not find the upregulation of PD-L1 and FasL (Figure 24, panel B).

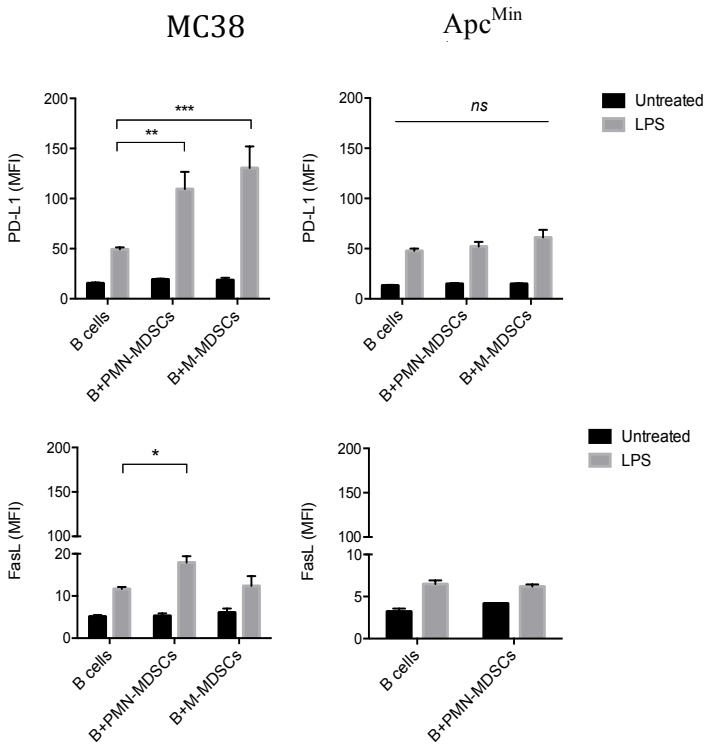


Figure 24. Induction of PD-L1 and FasL in B cells by splenic $Apc^{Min/+}$and MC38 MDSCs. A. B. PD-L1 and FasL expression was detected on purified splenic $CD19^+$ B cells from healthy mice after co-colture with PMN-MDSCs and M-MDSCs respectively purified from the spleens of both MC38 tumor-bearing mice and $Apc^{Min/+}$ mice.
*Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

Among the several works that reported the presence of tumor promoting TIL-B, Shalapur and colleagues revealed the presence of immunosuppressive plasmocytes expressing IgA, IL-10 and PD-L1 that conferred resistance to oxaliplatin treatment in the TRAMP mice. The authors demonstrated that the elimination of these cells allows the eradication of oxaliplatin-treated resistant tumors (Shalapour et al., 2015).

An additional recent work demonstrated the ability of TIL-B to favour CRC progression in MIR15A and MIR16-1 knockout mice treated with AOM/DSS. Also in this work, most of the B cells in the tumor were IgA^+ and expressed high levels of PD-L1, IL-10, TGFβ and repressed the proliferation of $CD8^+$ T cells (Liu et al., 2018).

In light of these data, I decided to assess the ability of splenic MC38-derived MDSCs to induce IgA on healthy splenic B cells. As for the expression of PD-L1 and FasL, MDSCs were also able to induce IgA expression on B cells in presence of LPS (Figure 25).

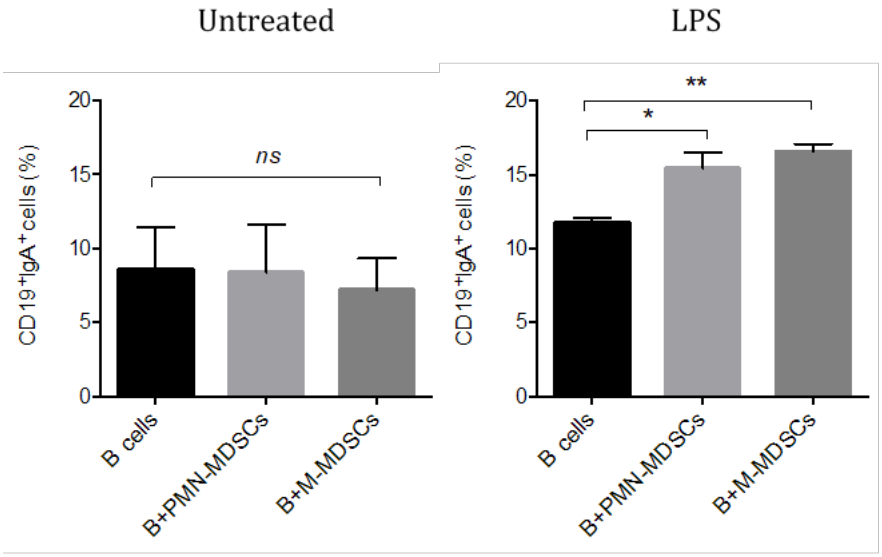


Figure 25. MC38 splenic MDSCs induce IgA on B cells. IgA expression was assessed by flow cytometry on $CD19^+$ B cells purified from healthy spleens after co-culture with PMN-MDSCs and M-MDSCs purified from the spleens of MC38 tumor-bearing mice. *Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001; ns: not significant.*

Collectively, we demonstrated that MDSCs could contribute to educate naive B cells to acquire the expression of IL-10, IgA, FasL and PD-L1 markers. In literature, this phenotype has been associated with a tumor-promoting population of B cells, therefore I hypothesize that, in the CRC context, MDSCs educate B cells to become immunosuppressive and to favour the CRC progression.

4. DISCUSSION

In the last decade, although the research have been done large advancements in the diagnosis and therapy of CRC, this disease remains the fourth most common cause of cancer-related death worldwide (Gandomani et al., 2017).

The TME covers a considerable importance in the tumor progression, therefore, a detailed investigation of the TME and the characterization of the crosstalk with the immune system could support the discovery of new therapeutic approaches for the treatment of CRC.

The mucosal surfaces of the gastrointestinal or respiratory tracts form vast interfaces of the host, have a selective permeability, are responsible for nutrient absorption and prevent the entry of pathogens. The mucosal immune system is a complex network of cells and molecules responsible for the protection of our body.

The secretory-IgA (SIgA) serves as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (Corthésy, 2013).

It has been already demonstrated an association between elevated levels of serum IgA and human CRC, (Baseler et al., 1987) and, more recently, Mion and colleagues underlined the involvement of the IgA response in the colon adenomatous transformation in the $Apc^{Min/+}$ mice (Mion et al., 2017).

However, the switch regulation and the role of IgA in the CRC have still not been investigated. In the present work, we aimed to investigate the involvement of this Ig in the onset and development of CRC.

One of the most inteseting *in vivo* model to investigate the intestinal tumorigenesis and the development of the CRC is the $Apc^{Min/+}$ mouse; in fact, the heterozygotes of this strain develop anemia and are highly susceptible to spontaneous intestinal adenoma formation. Although the $Apc^{Min/+}$ mouse has been of keen interest for studying intestinal tumorigenesis mechanisms; nevertheless, it has been reported that the *Apc* tumor suppressor gene plays a key role in the transduction of the *Wnt-signaling* pathway. In particular, it has been also

demonstrated that the defective *Wnt* signaling characterizing $Apc^{Min/+}$ mice influences the development and differentiation of several tissues, including haematopoietic tissues (Coletta et al., 2004; You et al., 2006),

In order to exclude that the altered levels of IgA were dependent on the mutations in *Apc* in different tissues rather than the gut, a different model of spontaneus colon cancer was investigated. We analyzed the $Cdx2^{Cre}Apc^{flox/flox}$ mouse model in which the *Cre* recombinase is under the control of the gut-specific *Cdx2* transcription factor (Hinoi et al., 2007). In these mice the *Apc* gene is deleted only in the intestinal epithelium and, unlike the $Apc^{Min/+}$ mice, these mice develop tumors primarily in the distal colon, providing a relevant model of human CRC.

Ig assays confirmed the increase only of the IgA levels in the $Cdx2^{Cre}Apc^{flox/flox}$ mice indicating that the CRC is accountable for raising of IgA levels (figure 9).

Interestingly, the analyses of serum levels of Ig and Ig released by B cells purified from different compartments (from different CRC models, i.e. AOM/DSS, CT26-FL3, and MC38 tumor-bearing mice) and *in vitro* stimulated, highlighted that sole IgA skewing is typical only of the $Apc^{Min/+}$ mouse model (figure 11, 12).

Indeed, all the other CRC models presented an overall change in the Ig balance compared to the healthy mice. However, in tumors unrelated to the intestinal tract, such as TRAMP mice, mice orthotopically injected with the KP cell line and in d16HER2 breast cancer tumor-bearing mice, compared to their healthy controls, there are no changes in IgA levels (figure 13). In conclusion, the observed data indicated that the IgA skewing constitutes a specific feature of the CRC mouse model $Apc^{Min/+}$, suggesting a strong role of the tumor macroenvironment in the activation of the B cell immune response also at distal sites.

Furthermore, data supporting our study have been also reported in humans: Chalkias et al., have, in fact, underlined the presence of elevated serum level of IgA in human patients with colon cancer (Chalkias et al., 2011).

Intestinal diseases are often associated with increased intestinal permeability (Buhner et al., 2006). The disruption of the gut barrier allows microbial products and bacteria to translocate from the intestinal lumen to extra-intestinal organs. In this scenario, we hypothesized that during CRC progression, the intestinal barrier can be damaged leading to the translocation of microbial products, which can

facilitate tumor progression. In this work, the microbial translocation was evaluated through *in vitro* experiments in which homogenized livers from $Apc^{Min/+}$ and AOM/DSS mice were cultured and the bacterial growth was evaluated in comparison with those of the healthy controls. Given the increased intestinal permeability associated with gut diseases, we expected to find an increased concentration of translocated bacteria in the near organs, such as the liver.

In accordance with our hypothesis, the presence of a higher bacterial level in the $Apc^{Min/+}$ mice, but not in the AOM/DSS tumor-bearing mice, was found (figure 14).

To verify the obtained data, the 16S sequencing was performed in the bacterial DNA extracted from the whole blood of $Apc^{Min/+}$ and WT mice. The data confirmed the presence of a higher bacterial content in the $Apc^{Min/+}$ samples compared to the WT and the presence of a vast variety of bacterium in the $Apc^{Min/+}$ samples compared to those present in the WT mice (figure 15). These data are in accordance with the literature that extensively shows that bacterial translocation occurs in colorectal cancer, both in mice (Puppa et al., 2011) and patients (Chin et al., 2007).

During CRC progression, the tumor environment leads to the change in the bacterial composition; the intestinal niche alterations can, in fact, favour the proliferation of opportunistic bacteria. It has been recently reviewed that CRC can be initiated by "driver bacteria", that can be responsible for the epithelia DNA damage; successively these bacteria can be replaced by "passenger bacteria" that can either promote or block tumor progression (Tjalsma et al., 2012). In this work, the data obtained from the liver cultures indicate the abundance of the anaerobic bacteria *Streptococcus spp*. and a slight increase of *Escherichia Coli*, while the presence of *Bacteroides fragilis* and *Parabacteroides diastonis* was peculiar only of the $Apc^{Min/+}$ mice, resulting instead absent in the WT mice (figure 15). These data indicate the carcinogenic potential of the microbiota in the tumor context compared to the healthy mucosa, in fact, strong experimental evidence demonstrated the tumorigenicity of both *E. coli* and *B. fragilis* (Arthur et al., 2012; Wu et al., 2009).

Our data coincide also with the hypothesis that was recently proposed by Sears and Pardoll that suggests that some toxigenic bacteria, such as the *Bacteroides fragilis* are pro-oncogenic and able to model the mucosal immune response to further promote the CRC (Sears and Pardoll, 2011). This hypothesis could therefore explain and link the microbial translocation with the remodeling of the mucosal immune response that culminates in the elevated IgA response observed in the $Apc^{Min/+}$ mice, supporting, in turn, our hypothesis about the thinkable correlation between the altered intestinal permeability and the increased IgA production.

Furthermore, our hypothesis is also supported by a recent work in which the colonic mucosa of patients with FAP was studied (Dejea et al., 2018). The authors, in accordance with our data, identified bacterial biofilms composed predominately of *Escherichia coli* and *Bacteroides fragilis*. They also revealed the presence in FAP patients of increased IgA levels and confirmed the specificity of the immunoglobulins against the enzymes responsible for the DNA damage (Dejea et al., 2018).

Contrarily to the obtained data in the $Apc^{Min/+}$ mice, in the AOM/DSS mouse model no substantial differences were found in the tumor-bearing mice compared to the healthy control mice.

In the tumor context, an increasing amount of data demonstrated the correlation between the large accumulation of MDSCs with the modulation of B cell differentiation and function (Xu et al., 2017). Our data confirmed the accumulation of MDSCs in different setting of CRC and we found a different distribution of the myeloid subsets in the analyzed CRC context (figure 18, panel A, B). We also focused on clarifying the B/MDSC crosstalk in the tumor context and on elucidating the eventual effects of MDSCs in influencing the B cells phenotype and functions in the CRC setting.

In the last decade, a growing body of evidence highlighted the presence of an immunosuppressive subset of B cells in several contexts (Lundy, 2009; Mauri and Bosma, 2012; Olkhanud et al., 2011). These cells are collectively called B_{reg} and act mainly through the release of the regulatory cytokine IL-10 (Rosser and Mauri, 2015), that is fundamental in the control of inflammation. Despite Breg

cells were described both in healthy and pathological settings, the precise mechanisms responsible for their induction are still debated. In this work, we demonstrated the ability of splenic PMN-MDSCs purified from $Apc^{Min/+}$ mice, AOM/DSS and tumor-bearing mice co-cultured with naïve B cells isolated from the healthy control spleens, to induce the expansion of IL-10 competent B cells in presence of LPS (figure 21). We also highlighted that the release of IL-10 in the co-culture supernatants were not increased in presence of MDSCs (figure 22). The obtained data are not in contrast, in fact the expansion of IL-10–competent B cells and the production of IL-10 are two differentially regulated processes (Mion et al., 2014). We suppose that MDSCs, although do not induce the release of IL-10, could prepare and instruct B cells to become competent for a suppressive function when an appropriate and context-specific second signal will be encountered.

In addition to the mechanism driven from the anti-inflammatory cytokine IL-10, we also demonstrated the ability of splenic MDSCs isolated from MC38 tumor-bearing mice to induce the expression of the markers FasL, PD-L1, and IgA in presence of LPS stimulation (figure 24, 25). This data are in accordance with the recent data that demonstrated the presence of a specific subset of B cells resistant to the oxaliplatin treatment in the context of prostate cancer. The authors revealed that in TRAMP mice the resistant population of B cells was characterized by the expression of high levels of IL-10, PD-L1 and IgA (Shalapour et al., 2015). In support to our results, Liu and colleagues demonstrated the presence of suppressive B cells in the AOM/DSS tumor-bearing mice characterized by the expression of IgA, PD-L1, IL-10 and TGFβ (Liu et al., 2018). The data of FasL induction on B cells by MDSCs is sustained in several works that highlight the role of the expression of death ligands in the evasion of the immune surveillance. FasL expression on B cells was in fact associated with a poor prognosis in aggressive lymphoma and in B-CLL patients (Mullauer et al., 1998; Tinhofer et al., 1998); on the contrary B cell diseases expressing lower levels of FasL were less aggressive (Grüllich et al., 2003).

The presented data lead us to hypothesize that, in the CRC context, the accumulated MDSCs are able to educate B cells to acquire an immunosuppressive phenotype that could drive the CRC progression.

Collectively, the data presented in this thesis give a clearer visual on the mechanisms responsible for the increased IgA levels found in the $Apc^{Min/+}$ mice. We suppose that the increased IgA levels in the $Apc^{Min/+}$ mice could be linked with the presence of the bacterial translocation to the liver, caused by the gut barrier damage. We also underlined the consequence of the microbial translocation found in the $Apc^{Min/+}$ mice that resulted in the altered bacterial genus present in the $Apc^{Min/+}$ mice liver compared to the healthy liver. Furthermore, the altered bacterial distribution stressed the presence of some toxigenic bacteria, such as the *Bacteroides fragilis* only in the CRC mice that could promote the cancer progression. In the present work, we also highlighted a further mechanism occurring in CRC that could support the tumor progression. We demonstrated the presence of a large accumulation of MDSCs in the CRC environment and the ability of splenic MDSCs from MC38 tumor-bearing mice to affect the phenotype of B cells, inducing a shift towards an immunosuppressive B cell phenotype.

In conclusion, this work tried to clarify the changes of the tumor micro- and macroenvironment occurring in CRC and can justify the development of new potential immunotherapeutic strategies.

5. MATERIAL AND METHODS

5.1. Animal studies

C57BL/6 mice were purchased from Envigo+++, $Apc^{Min/+}$ mice and CT26-FL3 tumor bearing mice were kindly provided by Stefania Vetrano (Humanitas, Milano) and the $Cdx2^{Cre}Apc^{flox/flox}$ mice were provided from Professor Sergei Grivennikov (Fox Chase Cancer Centre, Philadelphia, USA). The TRAMP, KP lung cancer and the breast cancer d16HER2 samples were respectively provided by Mario Colombo (Department of Experimental Oncology and Molecular Medicine, Molecular Immunology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milano), Federica Benvenuti (ICGEB, Trieste) and Gustavo Baldassarre (CRO, Centro di Riferimento Oncologico, Aviano).

All animal experiments were performed in accordance with the animal care and use committees of the respective institutes.

5.2. Culture media and solutions:

All the experiments were performed using the complete culture medium:

- RPMI 1640 (Euroclone)
- 10% FBS (Sigma Aldrich)
- 20 mM Hepes (Euroclone)
- 2 mM L-glutamine (Euroclone)
- 1 mM sodium pyruvate (Euroclone)
- 1X non-essential amino acids (from 100X mix, Euroclone)
- antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone)
- 50 mM β-mercaptoethanol (Sigma Aldrich)

The MC38 culture, before cell injection, was performed using the DMEM culture medium:

- DMEM (Euroclone)
- 10% FBS (Sigma Aldrich)

- 2 mM L-glutamine (Euroclone)
- antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone)

The solutions used in this work are reported in the table 2.

Wash medium	RPMI 1640 (Euroclone); 10% FBS (Sigma Aldrich); 20 mM Hepes (Euroclone); 2 mM L-glutamine (Euroclone); antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone)
Phosphate Buffered Saline (PBS) pH 7.4	137 mM NaCl (Sigma Aldrich); 27 mM KCl (Sigma Aldrich); 4.3 mM Na_2HPO_4 (Sigma Aldrich); 1.4 mM KH_2PO_4 (Sigma Aldrich)
MACS buffer	PBS pH 7.4; 2 mM EDTA (Sigma Aldrich); 0.5% BSA (Sigma Aldrich)

Table 2. Used solutions for the present work.

5.3. Murine cell isolation

- **Splenic and LN B cells**

  B cells were isolated respectively from spleens and mesenteric lymph nodes of female C57BL/6 mice. The organs were mechanically disrupted and the erythrocytes fraction of the spleen was depleted by hypotonic lysis with ACK lysing buffer (SIGMA-Aldrich). B cells were isolated with B cell isolation kit, mouse (Miltenyi, 130-090-862) following the manufacturer's instructions, the obtained purity is around 95-99%.

- **Peritoneal B cells**

  B cells from the peritoneal cavity were obtained after peritoneal lavage performed with cold PBS supplemented with 3% of fetal bovine serum (FBS) (SIGMA-Aldrich). Briefly, after cutting the skin above the

abdomen, 5-10 ml of the solution are injected with a syringe in the peritoneal cavity and cells are push into suspension through a gentle massage. B cells were isolated using the CD19 microbeads, mouse (Miltenyi, 130-052-201).

Purified B cells were cultured at the final concentration of 1x106 cell/mL, in the presence or absence of 1 µg/mL anti-mouse CD40 mAb (BD Pharmigen), 10 µg/mL LPS (Sigma-Aldrich) or 5 µg/mL CpG (Sigma-Aldrich).

- **Splenic MDSCs**

  MDSCs were isolated from tumor spleens of C57BL/6 female mice. Spleens were mechanically disrupted and the erythrocytes fraction was depleted by hypotonic lysis with ACK lysing buffer (SIGMA-Aldrich). MDSCs were isolated with Myeloid-Derived Suppressor Cell Isolation Kit, mouse (Miltenyi, 130-094-538) following the manufacturer's instructions; the obtained purity is around 90-95% for PMN-MDSCs and about 80% for M-MDSCs.

  Purified MDSCs were co-cultured with B cells (1:1 ratio), in the presence or absence 10 µg/mL LPS (Sigma-Aldrich).

- **Isolation of MC38 tumor cells**

  MC38 tumors were collected and suspension cells were obtained by digesting the mass with 0.25 mg/ml Collagenase type IV (Sigma-Aldrich) and 5 U/ml DNase (Roche) in RPMI supplemented with L-glutamine and antibiotics at 37°C and 5% CO_2. FBS was added to stop the reaction after 30-45min. Cells, filtered through 70 µm-pore-size nylon filters, were resuspended in fresh complete RPMI medium and used for flow cytometry analysis or plated into cell culture multi-well plates.

5.4. Proliferation assay

B cells were marked with 5 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen-Molecular Probes) by incubating them for 15 minutes at 37°C. The reaction is blocked with FBS (SIGMA-Aldrich) and cells are co-cultured with MDSCs in a 1:1 ratio in the presence of 10 µg/mL LPS (Sigma-Aldrich) or in presence of 1 µg/mL of anti-mouse CD40 mAb + 20 µg/ml of anti-IgM (BD Pharmigen).

The proliferation rate was evaluated by flow cytometry after 72 hours. The adopted gating strategies is reported in figure 27.

5.5. Mouse tumor models

- **Subcutaneous MC38:** the MC38 cell line derived from methylcholanthrene-induced C57BL/6 murine colon adenocarcinoma cells was maintained in DMEM, at 37 °C under 5% CO_2 pressure. A total of $0{,}2 \times 10^6$ MC38 colon carcinoma cells were injected subcutaneously (s.c.) into the left flank of 9-week-old female wild-type mice. Tumor growth was monitored three times per week by measuring tumor length and width. Tumor volume was calculated according to the following equation: $½(\text{length} \times \text{width}^2)$. Mice were scarified and the organs processed when the tumors reached the length limit of 15 mm.

- **$Apc^{Min/+}$:** in this work, 10- and 18-weeks old female $Apc^{Min/+}$ mice, together with age- and gender-matched WT littermates, were used.

- **$Cdx2^{Cre}Apc^{flox/flox}$:** in this work, 5 month old female $Cdx2^{Cre}Apc^{flox/flox}$ mice, together with age- and gender-matched WT littermates, were used.

- **CT26-FL3:** this metastatic CRC mouse model was obtained by the cecal implantation of the Balb/c-derived mouse colon carcinoma cell line CT26 in Balb/c mice.

- **AOM/DSS:** C57BL/6 mice (4–6 weeks-old) were weighed and given a single intraperitoneal injection of azoxymethane (AOM; 10 mg/kg) or vehicle (PBS). Seven days later, animals received either 2.5% DSS or normal drinking water, respectively. Chronic colitis and colon cancer were induced after three cycles of DSS treatment, which consisted of 5 days of 2.5% fresh DSS followed by 14 days of normal drinking water. In order to validate the adopted protocol, the mouse weight was monitored to check the inflammation effects as reported in the figure 26. After the sacrifice, also the gut histological analyses were performed to validate the presence of the tumor.

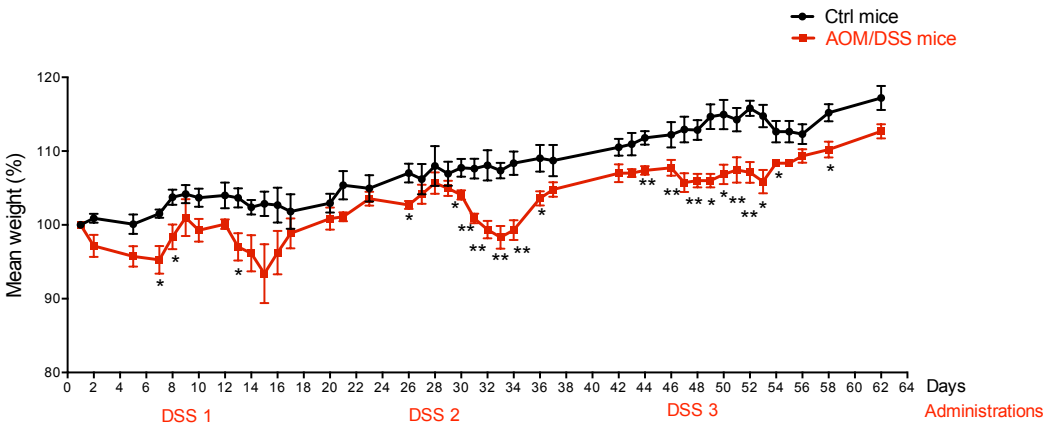


Figure 26. Weight monitoring between the AOM/DSS treated mice and the healthy controls. The weight of the mice was monitored every two days during the treatment. *Mean ± SEM are shown *p<0.05; **p<0.01; ***p<0.001.*

5.6. ELISA

Immunoglobulin and cytokine quantification in cell supernatants and sera was performed using the Enzyme Linked Immunosorbent Assay (ELISA), a highly sensitive and easy to use biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.

The ELISA experiments performed for this thesis are "sandwich ELISAs" in which the plate is coated with a capture antibody and, after an o/n incubation and the block of nonspecific binding sites on the surface, sample is added and any antigen present binds to the capture antibody. Then enzyme-linked detecting antibody is added and binds to antigen and, at last, a substrate is applied and is converted by the enzyme to detectable form.

In this work we used the IL-10 ELISA kit (eBioscience), the IgM ELISA kit (Thermo Fisher Scientific) that were used according to the manufacturer's instructions. The IgA and IgG ELISA were home made performed. 96-well flat-bottom polystyrene plates (Corning) were coated with affinity-purified anti-mouse IgA (SouthernBiotech) or anti-mouse IgG (Sigma-Aldrich) Abs at the final concentration of 2 mg/mL and 10 mg/mL, respectively. After 1h incubation at 37°C, plates were washed with 0.05% Tween-20 in PBS and blocked with 1% bovine serum albumin in PBS for 1 h at RT. 100 mL of cell supernatants or of opportunely diluted mouse sera were added to Ab-coated wells. Purified mouse IgA (BD PharMingen) or IgG (Sigma-Aldrich) were used as standards. After overnight incubation at 4°C, the plates were washed and optimal concentration of horseradish peroxidase-conjugated goat anti-mouse IgA (SouthernBiotech) or goat anti-mouse IgG (Pierce) Abs were added. Next, the plates were incubated for 1 h at RT and washed before the addition of tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich). The reaction was stopped with 2 mol/L sulfuric acid and absorbance was measured at 450 nm.

5.7. Bacterial DNA extraction

Whole blood samples were subjected to the DNA purification using the QIAamp Cador Pathogen Mini Kit (QIAGEN, 54104) following the manufacturer's instructions. The concentration of DNA was determined using the NanoDrop spectrophotometer.

5.8. Metagenomic analysis

For the metagenomic analysis the 16S Barcoding Kit (SQK-RAB204 Oxford Nanopore) was used.

The library was prepared using the SQK-MAP005 kit according to the manufacturer's instructions (Oxford Nanopore). Then, the MinION sequencing library was loaded into the MinION Flow Cell and the sequencing run was started.

Raw sequencing data were generated as raw FAST5 by MinKNOW software Agent and automatically uploaded to the Metrichor Agent cloud-based service. In order to analyse the data derived from MinION sequencing, the web-based platform Galaxy was adopted.

5.9. Bacterial translocation

The whole liver was homogenized in 3 ml of sterile PBS. Serial dilutions were then plated in different culture media and incubated at 37 °C under 5% CO_2 pressure and in anaerobic condition for 36 hours and CFU were enumerated. The used media are reported in the table 3.

Media	Culture condition
Brucella	Anaerobic
TSA	Aerobic
Orientation	Aerobic
Chocolate	Aerobic
LB	Aerobic

Table 3. Media used for bacterium growth.

5.10. Flow cytometry analyses

To assess cell-surface expression of different phenotypic and activation markers or co-stimulatory molecules, cultured cells were collected into polystyrene tubes

(Sarstedt), washed and resuspended with PBS. After the addition of fluorescent mAbs, or Ig isotype-matched controls, cells were incubated in darkness for 30 minutes at 4°C. After the incubation, cells were washed with PBS and flow cytometry analyses were performed. FACSCalibur (Becton Dickinson) was used for sample acquisitions. A list of the antibodies used in this work is shown in table 4.

Specificity of Antibody	Clone	Manufacturer
CD11b	M1/70	BioLegend
Gr-1	RB6-8C5	BioLegend
Ly6C	HK1.4	BioLegend
Ly6G	1A8	BioLegend
CD19	6D5	BioLegend
CD4	RM4-5	BD Pharmigen
FasL	MFL3	eBioscience
PD-L1	10F.9G2	eBioscience
IgA	C10-3	BD Pharmigen
IL-10	JES5-16E3	BioLegend

Table 4. Antibodies used for immunophenotyping.

5.11. Intracellular staining for IL-10 detection

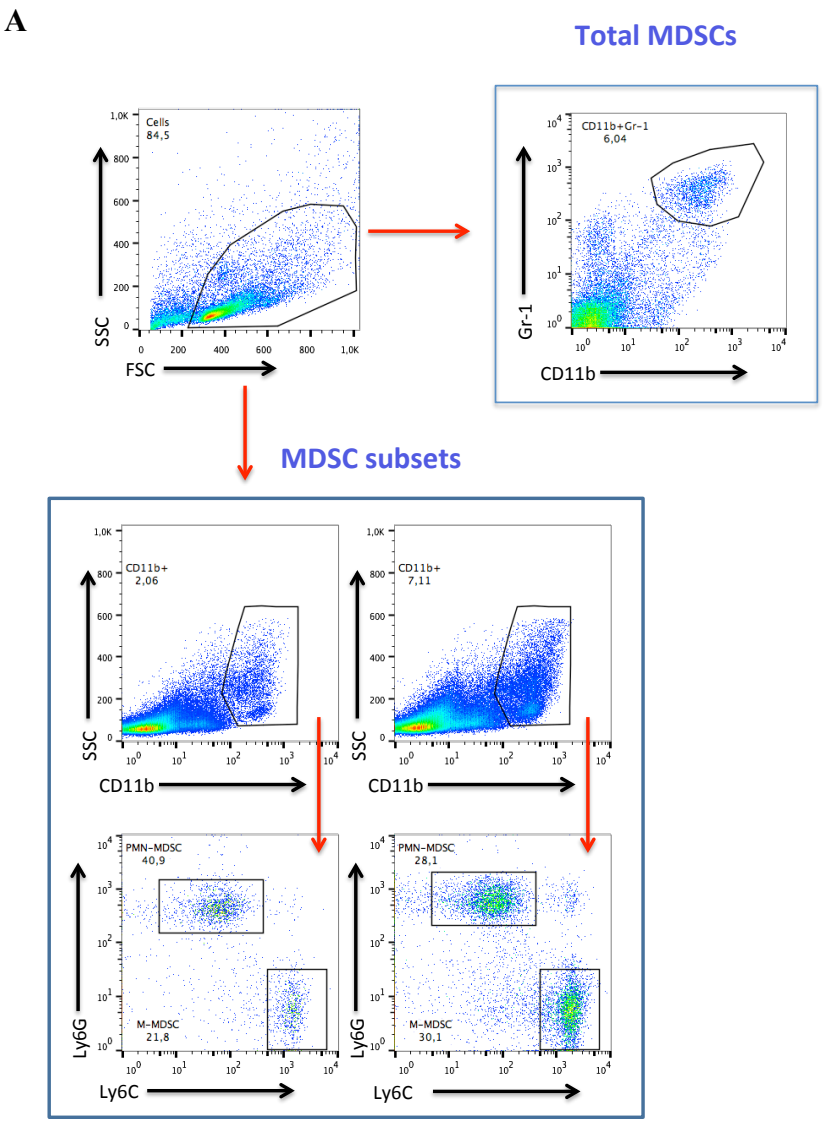
For immunofluorescent staining of intracellular IL-10, B cells are resuspended at $1x10^6$ cells/ml in culture medium containing 50 ng/ml PMA (SIGMA-Aldrich), 500 ng/ml ionomycin (SIGMA-Aldrich), 10 µg/ml LPS (SIGMA-Aldrich) and 2 µM monensin (eBiosciences) and cultured for 5 h at 37°C and 5% CO_2. At the end of the 5 h of stimulation cells are collected and washed with PBS before being stained with the green fluorescent probe LIVE/DEAD Fixable Green Dead Cell Stain Probe (Molecular Probes, Life Technologies) in order to discriminate viable cells from dead ones. Subsequently, the anti CD16/CD32 mAb is added to avoid non-antigen-specific binding of Fc portion of antibodies. The two reagents are incubated together for 15 min after which cells are washed and incubated for 30 min with the CD19 mAb (BioLegend). After surface staining, cells are fixed with

250 µl of Cytofix/Cytoperm cell fixation buffer (Becton Dickinson) for 20 min and then washed two times with Perm/Wash buffer (Becton Dickinson) for cell permeabilization. Hereafter Perm/Wash buffer is used for staining and washes in order to maintain cells in a permeabilized state. Cells are resuspended in Perm/Wash buffer containing PE-labeled anti-IL-10 mAb for 30 min. Finally, cells are washed twice with Perm/Wash buffer and resuspended in 250 µl of 1.5% formaldehyde fixative. Cells were kept at 4°C until analyzed by flow cytometry.

5.12. Gating strategies

The adopted gating strategies are reported in the figure 27.

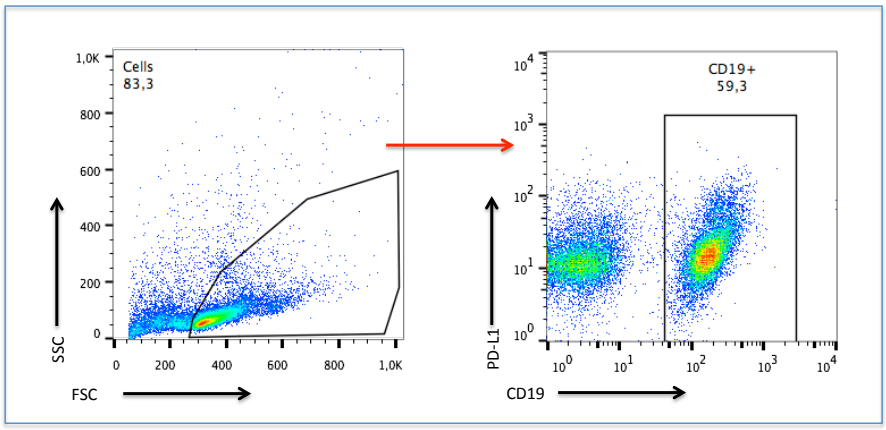
- **MDSCs**



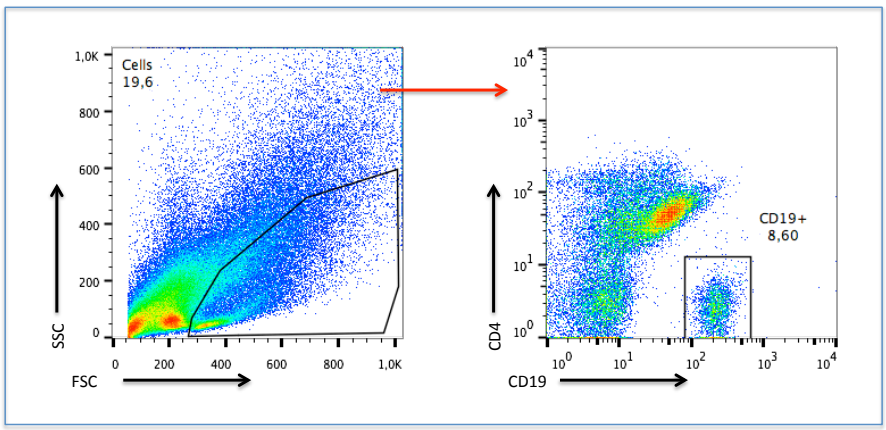
- **B cells**

B

Splenocytes



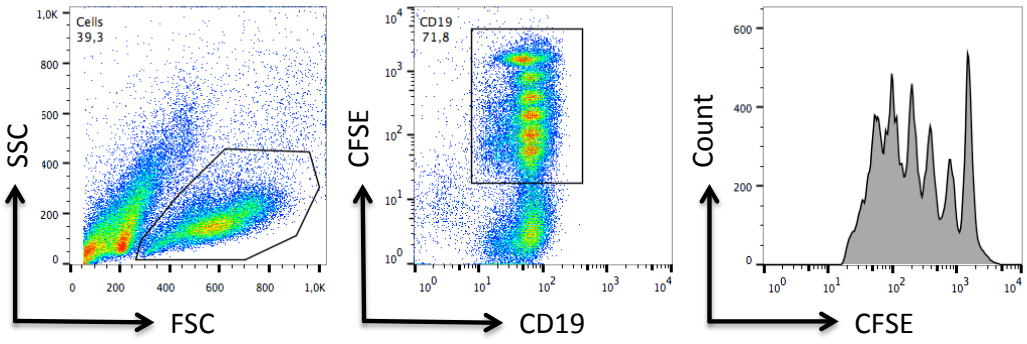
Tumor cells



- **Proliferation assay**

C

B+PMN-MDSCs



- **IL-10 ICS**

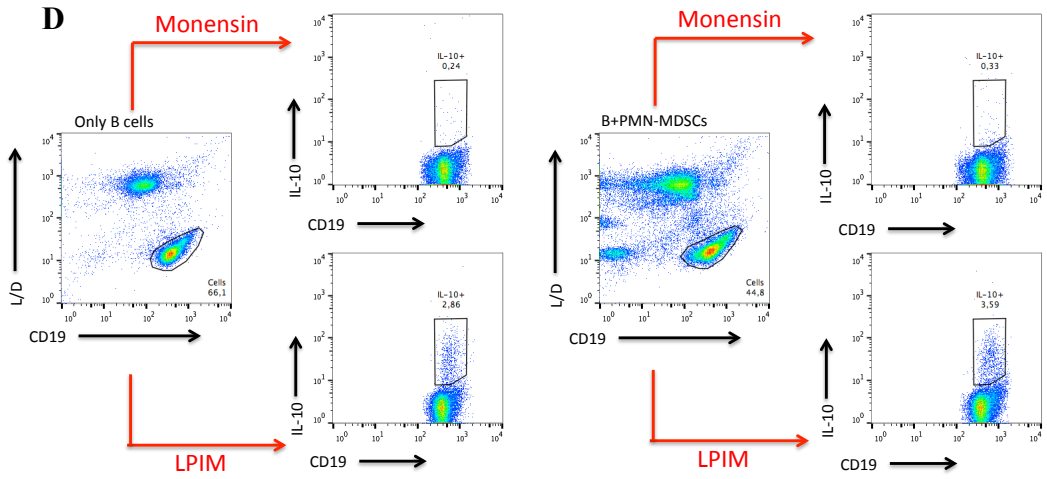


Figure 27. Adopted gating strategies for flow cytometry analyses. A. The total MDSCs were first gated in the FSC vs SSC plot. Cells are further selected for CD11b and Gr-1 positivity. PMN-MDSCs and M-MDSCs were gated in the FSC vs SSC plot and then selected for CD11b positivity. The percentage of PMN-MDSCs and M-MDSCs were then respectively obtained on gated $Ly6G^+$ and $Ly6C^+$ cells. **B.** B cells were first gated in the FSC vs SSC plot. Both in the spleen and tumor samples. Cells are further selected for CD19 positivity and on these gated cells, second membrane markers were analysed.
C. B cells were gated in the FSC vs SSC plot and the analyses of $CFSE^+$ cells were performed on gated $CFSE^+CD19^+$ cells. **D.** B cells were gated on L/D^- cells and on $CD19^+$ cells. The analyses of $IL\text{-}10^+$ cells were then performed respectively on monensin and LPIM treated cells. In the right panel an example of the same analysis of B cells in the co-culture with PMN-MDSCs is reported.

5.13. Statistical analyses

Experimental data are shown as mean +/- standard error of mean (SEM). The unpaired or paired Student's t-tests, the ANOVA test and the Spearman Test (Prism, GraphPad Software, La Jolla, CA, USA) were used to analyse the results for statistical significance. P values below 0.05 were considered as significant.

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