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Cellular and molecular mechanisms of immunosurveillance escape in Head Neck Squamous Cell Carcinoma

Meccanismi cellulari e molecolari coinvolti nell'evasione dall'immunosorveglianza nei carcinomi testa-collo a cellule squamose

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Introduction

The Immune System

The immune system is the collection of cells, tissues and molecules that protects the body from numerous pathogenic microbes and macromolecules as proteins or polysaccharides. All the molecules capable of inducing immune response are addressed as Antigens. Sometimes they can be part of the host itself and give rise to autoimmune diseases.

The defence against pathogens is divided into two types of responses (1):

- Innate Immune responses;
- Adaptive Immune responses.

Innate Immunity

Most of the Innate Immunity components shows high similarity in different species thus confirming that innate immune system is the phylogenetically older defense mechanism that evolved together with pathogens in old ancestors.

Innate immunity provides first line defence to pathogens and cooperates together with adaptive immunity providing signals of the ongoing infection and stimulating specific response mechanisms.

Innate Immune defences differ from adaptive ones because they are non-specific and do not confer long-lasting immunity against a pathogen.

Innate immunity first-line defense against microbes are physical barriers, while several recognition receptors expressed by Innate cells permit pathogen recognition and elimination.

Physical Barriers

The Epithelial surfaces integrity represent a physical barrier that isolate host tissue from infectious agents. Among the mechanical anatomical barriers, we found:

- Skin

- Gastrointestinal tract

- Respiratory airways and lung

- Nasopharynx

- Urogenital tract

Moreover, skin desquamation, movement of the intestines and the oscillation of broncho-pulmonary cilia helps to remove pathogens.

In addition to these protective surfaces, epithelial cells produce:

- Mucus: a slippery secretion containing glycoproteins. Majors' mucus producing cells are respiratory, gastrointestinal and urogenital epithelial cells. Mucus provides a trapping effect that lines the respiratory and gastrointestinal tract in order to protect the lungs and digestive systems from infection.

- Antimicrobial peptides as Defensins and Catelicidins. Defensins are low molecular weight proteins, produced by mucosal epithelial cells, as Paneth cells in the intestinal crypts, and leucocytes: Neutrophils, NK, T lymphocytes. Cathelicidins produced as precursors are further activated by proteolysis. The main source of cathelicidins are neutrophils, but they can be also produced by epithelial cells in different districts including respiratory and gastrointestinal mucosa (1).

Epithelial integrity alterations, as mechanical injury, leads to inflammatory mediators release such as IL25, IL33 and TSLP. These cytokines are also called "alarmins" and provide a link between tissue damage/ injury and immune

responses. Thus, airway epithelial cells are no longer considered a mere barrier but may play a pivotal role in regulating the immune responses and inflammation (2).

Innate Cells

Part of the innate immune responses are mediated by different type of cells, called Leukocytes, enhancing the protection offered by anatomical and physiological barriers. Leukocytes are not tightly associated with a particular organ or tissue; thus, their function is similar to that of independent, single-cell organisms. Leukocytes are able to move freely, interact with and capture cellular debris, foreign particles, and invading microorganisms (3).

The Innate leukocytes include: Natural Killer cells (NK), Mast cells, Eosinophils, Basophils, the recently discovered Innate Lymphoid Cells (ILCs); and also, phagocyting cells like: Macrophages, Neutrophils, and Dendritic Cells (DCs) (4).

All these cells, exert different functions in order to protect the host from different pathogens.

Phagocyting cells: First line of defense in case of injury, as Macrophages and DCs are tissue-resident cells that exert the function of "patrol", while Neutrophils are recruited to the site within minute and are the hallmark of acute inflammation (1). These cells have the ability to phagocytize a pathogen, wrapping a portion of their membrane around it. Once inside the cell, the invading pathogen is contained inside a vesicle called phagosome, which merges with another vesicle, called lysosome, that contains enzymes and acids that kill and digest the particle or organism.

Furthermore, Macrophages and DCs are also called Antigen Presenting Cells (APC) due to their ability to process and present antigens to the cells of the Adaptive Immune compartment. Once the pathogen has been digested, some peptides are bound to molecules called class II Major Histocompatibility

Complex (MHC II), and the peptide-MHC II complexes are then transported to the surface and the processed antigens presented to other cells like T-lymphocytes. There is also a class I MHC (expressed by all the nucleated cells), in this case the peptides presented are generated endogenously by the proteasome. This latter mechanism enables the Immune System to detect, and then eliminate, cells infected by viruses or intracellular pathogens, but also transformed/mutated cells.

Natural Killer Cells: Originally described in the 1970s as large granular lymphocytes exhibiting "natural cytotoxicity" against several types of tumor cells (5). More recently, new members with distinct functional attributes have rejuvenated the innate lymphoid cell (ILC) family and great progress has been made in molecularly dissecting NK cell specificity and function (6). It is now appreciated that NK cells not only can kill neoplastic or malignant cells and contribute to control of intracellular pathogens, but also maintain immune homeostasis through killing of several types of activated immune cells. To carry out these functions NK cells are equipped with a molecular detection system that includes a variety of cell surface activating and inhibitory receptors that facilitate the discrimination of target cells (7). Activating NK cell receptors may combinatorially induce NK cell effector functions through sensing the presence of ligands that are upregulated on infected, distressed or activated cells. In parallel, NK cells use inhibitory receptors to monitor the presence of constitutively expressed self-MHC class I molecules; sparing cells that normally express MHC class I (8). NK cells are an integral component of the immune system. Through their molecular detection system, they may act as sentinels for detecting aberrant cells. Their activation does not only involve target cell killing through release of perforin- and granzyme-containing cytotoxic granules. In response to target cell engagement, NK cells can secrete interferon (IFN)-γ and tumor necrosis factor (TNF) (8). Moreover, they represent an effector arm of humoral immune responses, expressing the low-affinity, activating Fc receptor CD16 that can trigger potent effector functions.

Thus, NK cells may through secretion of soluble factors drive inflammation, shape and regulate the activities of other immune cells and by these means affect the development of adaptive immune responses (8).

Pathogen recognition

Innate immunity recognizes two main molecules structures:

1) Pathogen Associated Molecular Patterns (PAMPS): Pathogen characteristic molecular structures, absent in the mammalian cells, that belong to different types of microbes: virus, Gram-positive bacteria, Gram-negative bacteria and fungi

Some examples are:

- Viral ssRNA and dsRNA

- Viral or bacterial CpG non-metilated sequences

- Gram negative outer membrane lipids or carbohydrates as lypopolysaccaride (LPS)

- Gram positive cell wall components as Lipoteichoyc Acid

2) Damage associated Molecular Patterns (DAMPS): Endogenous molecules released by cells after an infection or mechanical damage leading to necrosis, apoptotic cells do not release DAMPS.

PAMPs and DAMPs recognition in Innate Immunity involves both cell receptors and soluble molecules (4).

PRR (Pattern Recognition Receptors)

PRR are the Innate Immunity receptors. They can be exposed on the plasma membrane or in the endosomal compartment of phagocytes (Neutrophils and Macrophages), Dendritic Cells and epithelial cells. PRR are germ line coded whereas T and B lymphocytes receptors undergo a somatic rearrangement, this explain why innate immunity can respond to a restricted PAMPs

repertoire (10^3) compared to adaptive immunity (10^7). That is due also to the low complexity displayed by microbial molecules, upon which immune system have been modelled.

PRR could be classified in different classes and based on their location, may be divided into two main groups, membrane-bound and cytoplasmic:

1) **Membrane-bound PRR**

- **TLRs (Toll-like Receptors)**: In humans have been described at least nine types of TLRs. The structure of this transmembrane protein consists in an extracellular leucine-rich domain accompanied by cysteine-rich domain, responsible for pathogen recognition; a cytosolic carboxyl terminal Toll-interleukin-one receptor domain (TIL) essential for cellular activation, shared also by IL-1 and IL-18 cytokine's receptors that activate similar signals pathways.

  According to their localization we identify intracellular and extracellular TLRs. TLR3, TLR7, TLR8 and TLR9 are intracellular mainly distributed in the endoplasmic reticulum an endosomal membrane. Others TLRs as TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on plasma membrane recognizing PAMPs (9).

- **CLRs (C-type lectin receptors)**: Comprise a large superfamily of proteins, which recognise a various range of ligands, carbohydrates mainly, and are defined by the presence of at least one C-type lectin-like domain. They can be clustered into two broad groups based on their signalling potential:

  1) The mannose receptor (CD206): This is predominantly presented on Macrophages and DCs surfaces and recognize terminal mannose, N-acetylglucosamine and fucose residues on glycans attached to proteins on the surface of some microorganisms. No activator functions were addressed to these receptors, as their primary function is to mediate phagocytosis.

2) Dectin- 1 (Dendritic Cell-Associated C-Type Lectin 1) or CLEC7A (C-type lectin domain family 7-member A): Dectine-1 is a transmembrane protein containing an intracellular tail is involved in cellular activation characterized by ITAM-like motif. It plays a role in innate immune response as pattern-recognition receptor for a variety of β-1,3-linked and β-1,6-linked glucans from fungi and plants and are expressed on myeloid DCs. Ligand binding induces dimerization and ITAM-like motif tyrosine phosphorylation by Src family kinases. The intracellular pathway leads to NF-kB activation and production of numerous protective antifungal cytokines that promotes Th17 effective phenotype. In fact, fungal ligands recognition triggers a type III response (1) (4).

- **Scavenger receptors:** Mainly concentrated in macrophages, driving the process of phagocytosis. This naming is due to their "scavenging" function since they recognize macromolecules as modified low-density lipoprotein (LDL) that acquired a negative charge upon oxidation or acetylation. They are categorized into two classes, according to their structural characteristics. Scavenger receptors type 1 (SR-A1) and 2 (SR-A2) belongs to the A class and are trimers with a collagen-like domain, which is essential for ligand binding. While CD36, class B scavenger receptor, concentrate in a specific plasma membrane structure, the caveolae. In addition, CD36 cooperate in lipoteichoic acid and deacetylated lipopeptides response serving as coreceptor in TLR2/6 heterodimer (4).

2) **Cytoplasmic PRR**

- **NOD (Nucleotide Oligomerization domain) receptors:** NLRs family, comprehends more than 20 different proteins that recognize cells stress products. NLRs present three different domains: C-terminal leucine-rich repeat (LRR) that senses the presence of ligand; a NATCH domain

(Neuronal Apoptosis Inhibitory Protein CIITA, HET-E, and TPI) mediates ATP-dependent self-oligomerization; an N-terminal effector domain that presents a great variability: it can consist of caspase recruitment domain (CARD), pyrin domain (PYD), acidic transactivation domain or baculovirus inhibitor repeats (BIRs). The N-variable domain is responsible for homotypic protein-protein interaction. Better-described NLR receptors are NOD1 and NOD2 that recognize peptidoglycan motifs. Both NOD1 and NOD2 are characterize by a CARD N-terminal domain, once activated they recruits RIP2 kinases that mediate the NF-Kb activation to promotes the inflammatory cytokines expression. The NLR subfamily named NLRP (NLR Family, Pyrin Domain-Containing-Proteins) responds to cytoplasmic PAMPs and DAMPs and is involved in the Inflammasome formation. The Inflammasome is an NLRP oligomer that recruits type 1 caspases an provides to IL-1β and IL-18 activation. In addition to NBD and LRR domain, NLRP contains at its N-terminal a PYD pyrin domain. Upon activation many NLRP interact and leads to oligomer formation recruiting the ASC adaptor to activate caspase-1 cascade that process IL-1β and IL-18 precursors (1) (4).

- **RLRs (RIG like receptors):** These intracellular pattern recognition receptors are mainly involved in virus recognition. They detect double stranded RNA to senses viral replication. Tissues cells together with innate cells presents RLRs thus providing a wide defense among the possible virus target cells. RIG-I (Retinoic Acid Inducible Gene) and MDA5 (Melanoma Differentiation Associated Gene 5) are two main RLRs receptors that induce IRF3 and IRF7 activation via CARD domains leading to IFN type I production.

Inflammation and Cytokines

At the onset of an infection or other injuries, the immune system provides a protective response that involves immune cells, molecular mediators and blood vessels. This is known as inflammation whose first aim is to eliminate the initial cause of injury, clear out necrotic cells, and to initiate tissue repair. Three major events occur during this response:

1. Vasodilatation and Increased perfusion of in the infected area
2. Retraction of endothelial cells and increased capillary permeability causing edema (exudation of plasma proteins and fluid into the tissue).
3. Circulating leucocytes margination and endothelium adhesion leading to extravasation

We can distinguish either an acute and chronic inflammation. The process of acute inflammation involves resident cells already present in the damaged tissue, as neutrophils, resident macrophages, dendritic cells and mast cells. If infection persist, a progressive change in the type of cells present at the site of inflammation, such as lymphocytes drives to chronic inflammation. Persistent efforts of healing, sustained angiogenesis and fibrosis process give rise to tissue remodelling.

The central event that trigger acute inflammation is cytokines release. Among pro-inflammatory cytokines, we count TNF-α, IL-1β and IL-6.

Tumor Necrosis Factor (TNF)

The name is due to the experimental system that led to its discovery: the induction of necrosis in tumours by injection of sera, containing factors, into the tumour-bearing animals. The major source of TNF are macrophages and DCs, but is also produced by a broad variety of cell types. We can distinguish TNF-α from TNF-β, the second one also knows as lymphotoxin. TNF-α mediates the inflammatory response to bacteria and the gene expression controlled by NF-kB is affected by TLR binding to Gram+ and Gram- related PAMPs and DAMPs, for example LPS and Lipoteichoic acid. TNF-α is

produced as type-II-transmembrane protein and is released via proteolytic cleavage by the metalloprotease TNF-α converting enzyme TACE, also called ADAM17. The soluble form TNF-α takes on a trimeric pyramidal structure that permits the simultaneous binding to three different receptor molecules. There are two different TNF-α receptors: *TNF receptor type 1* (TNFR1) is expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms. Whereas, TNF receptor type 2 (TNFR2) is typically expressed by immune cells. Upon ligand contact, the receptor cytoplasmic chains associate with an adaptor protein *Tumor necrosis factor receptor type 1-associated DEATH domain* (TRADD) thus leading to activation of different pathways.

Following the *TNF Receptor Associated Factors* TRAF association we can have either NF-kB activation and MAPK cascade that terminates with AP-1 activation. Instead, TRADD binding to *Fas-Associated protein with Death Domain* (FADD) recruits the cysteine protease caspase-8 that cleaves effector caspases, driving the cell to apoptosis (1) (4).

Interleukin 1 (IL-1β)

The human IL-1 gene family comprises IL-1α, IL1-β, and IL-1 receptor antagonist (IL-1ra). IL1β and TNF-α have many effects in common as they both mediates acute inflammation. Both IL-1α and IL-1β binds to the same receptors, in spite of their low amino acid homology sequence, and have the same functions, although IL-1β is the main active form. IL-1β is produced as a precursor, pro-IL-1β (33kDa), upon TLR and NOD activation cascade that culminates in NF-kB activation. Pro-IL1β is cleaved in the NLRP3 inflammasome and generate IL-1β (17 kDa). Otherwise, IL-1β can be secreted in a non-classic way after macrophages death. TNF-α stimulate IL-1β production by macrophages and this demonstrate the strict connection between these two pro-inflammatory cytokines. IL-1 cytokines exert their function through a family of receptors that belong to the Toll-like receptor-IL-

1 receptor (TLR-IL-1R) superfamily, which is defined by the presence of an intracellular Toll-IL-1R (TIR) module. Two distinct IL-1 receptors have been identified, both exhibiting three extracellular immunoglobulin domains, type I and type II IL-1R differs for the length of cytoplasmic domain. Type IL-1Rc includes TIR domain, type II is critically shorter and lacks this signal transduction sequences. The type I IL-1R is an 80-kDa glycoprotein, found predominantly on T cells and fibroblasts. IL-1 via Myd88 e IRAK4, leads to several transcription factors activation as Nf-kB and AP-1. As TNF, IL-1, stimulates the monocytes and neutrophils recruitment by enhancing the integrin/selectin expression and chemokine production as CXCL1 and CCL2. IL-1 receptor antagonist (IL-1Ra) regulates the IL-1 proinflammatory effects. IL-1Ra binds IL-1 receptors competitively without inducing any biological response. In addition, IL-1 activity is regulated by soluble type II IL-1 receptor (IL-1sRII), that acts at ligand level. It has been described that those two inhibitors abolish each other's effects (10).

Interleukin 6 (IL-6)

IL-6 is another important cytokine involved in the inflammatory acute response. IL-6 is produced by mononuclear phagocytes, endothelial vascular cells and fibroblast upon IL-1, TNF-α and PAMPs stimulation. IL-6 together with IL-1 and TNF-α, are produced in the site of inflammation but can lead to systemic effect. Once reached the bone marrow, those cytokines, stimulate neutrophil production, co-operating with CSF (*Colony Stimulating Factors*). Moreover, IL-6 in the liver induces the release of others inflammatory mediators triggering the inflammatory response. IL-6 receptor displays one short α chain that recognize the ligand and two β chains gp130 that form homodimer and are responsible for signal transduction that ends with phosphorylation of transcription factor STAT3.

Adaptive Immunity

The adaptive immunity is a sophisticate defense system that display peculiar characteristic:

- **Specificity and diversification**: Thanks to clonal receptors expressed by T cells repertoire, adaptive immune system discriminates between 10^7 and 10^9 different molecular epitopes (the parts of an antigen that interact with an antibody molecule or a lymphocyte receptor).

- **Clonal expansion**: Upon antigens recognitions, specific lymphocytes undergo rapid proliferation.

- **Specialization**: As the adaptive response retains high specificity, it also provides different specific response depending on the insult received.

- **Memory**: First exposure to an antigen generates long lasting memory lymphocytes that are highly efficient in antigen elimination once re-exposure occurs.

- **Homeostasis**: Lymphocytes expansion and survival expire upon antigen elimination.

- **Immunological tolerance**: Self-reactive lymphocytes elimination in order to maintain a state of unresponsiveness to self-antigens. We can distinguish two types of immune tolerance: central and peripheral.
  Central immune tolerance occurs during T lymphocytes maturation in the primary lymphoid organs thymus and bone marrow, for T and B lymphocytes respectively. In these tissues, maturing lymphocytes are exposed to self-antigen imported from the periphery or expressed by thymic stromal cells, by the action of the transcription factor AIRE. Lymphocytes that strongly bind to self-antigens are removed by induction of apoptosis. Peripheral tolerance develops once mature T and B cells enter the peripheral tissues and lymph nodes. This mechanism is complementary to the first one as it provides a second line of defense for those self-reactive

T cells that escape intrathymic negative selection, and moreover it offers a wider range of self-antigens than those expressed by AIRE in thymic tissues such those expressed in the islets of Langerhans, brain, or spinal cord (4).

There are two types of adaptive responses, mediated by different cells and molecules:

- Cell-mediated immunity
- Humoral immunity

Cell-mediated Immunity

Adaptive cell-mediated immunity is the immune response that involve antigen specific T lymphocytes, that provide protection releasing different cytokines or directly inducing pathogen death. T lymphocytes derive from the Common Lymphoid Progenitor and they are involved in cell-mediated Immunity responding to intracellular pathogens and viruses. Unlike innate immunity, where specific receptors are already encoded in the germline, pathogen-specific receptors are acquired during the lifetime of the organism by T cells. The main mechanism that allows a small number of genes to generate a vast number of different antigen receptors is the V(D)J recombination. This is an irreversible genetic recombination of variable region segments, where some sequences are deleted, in order to generate many different antigen receptors. One lymphocyte will uniquely express one T cell receptor (TCR), and the offspring of that cell (T cell clone) will inherit genes that encodes the same TCR. TCR is and heterodimeric protein and comprises the highly variable α β chains with the invariant CD3 chain molecules. T cells that express this receptor are referred to as $\alpha\beta$ T cells, though a minority of T cells express an alternative receptor, formed by variable γ δ chains, and those are referred to as $\gamma\delta$ T cells. The variable region of each α β chains, binds to a specific peptide/MHC complex during physical contact between T cell and Antigen Presenting Cell (APC). On the contrary, $\gamma\delta$ T cells binds selectively to lipids and non-protein antigens without the involvement of MHC complexes.

Although more studies are needed to better understand their role, γδ T cells, seem to show a lower diversification and are thought to hold their role at the earlier stages of defense, in the epithelial barriers (11). Invariant CD3 molecule complex includes a CD3γ chain, a CD3δ chain, and two CD3ε chains all characterized by a conserved motif known as Immunoreceptor Tyrosine-based Activation Motif (ITAM), in the intracellular tails. The CD3 complex, together with the ζ chain, provides the TCR signal transduction (1).

Two more molecules expressed on T lymphocytes surface, CD8 and CD4 co-receptors, define the specificity of the TCR to MHC class I or II molecule respectively. CD8 and CD4 also increase TCR binding affinity for MHC, prolonging the interaction with the APC (4).

Relying on the CD4 and CD8 selective expression we can distinguish two main family of T lymphocytes:

- CD4+ T lymphocytes
- CD8+ T lymphocytes.

CD4+ T Lymphocytes

This wide family comprises two main subpopulations from a functional point of view:

- Effector cells that provide protection against exogenous pathogens.

- Regulatory T (Treg) cells that are involved in the immune suppression to maintain self-tolerance.

The effector subset is classified in different lineage upon patterns of cytokines secretion, transcription factor, and homing receptors expression.

For many years since their discovery, only the T helper 1 (Th1) and the T helper 2 (Th2) subsets were thought to belong to effective T CD4+ cells (12) (13). More recently, other T helper populations have been defined. These include the Th17 subset together with Th22, Th9, and T follicular helper (Tfh) (14).

Th1 Lymphocytes

The cytokines involved in Th1 differentiation from naive CD4+ T cells are IL-12 and IFN- γ, produced by macrophages and NK cells respectively. Both IL-12 and IFN-γ, via signal transducer and activator of transcription 4 (STAT4) and STAT1, promote transcription of Th1 "master regulator transcription factor" T-bet, thus leading to IFN-γ production. IFN-γ amplifies Th1 differentiation while inhibits other T helper phenotype cells proliferation. At later stages of Th1 differentiation, IL-12/STAT4 signalling stimulate IL-18Ra upregulation. IL-18 and IL-12 are mainly produced by APCs and jointly induce direct IFN-γ production by Th1 cells in the absence of T-cell receptor stimulation. Moreover, these two cytokines are involved in an activation loop that amplify Th1 response. In fact, IL-18 produced by APCs stimulate NK cells to produce IFN-γ inducing naïve T cells to express IL-12 receptor β chain (the α chain is constitutively expressed), in order to enhance IL-12 dependent Th1 polarization (15). Furthermore, T lymphocytes can directly induce IL-12 production by APCs upon CD40L-CD40 binding (1). Th1 cells play an important role in the protection against intracellular pathogens. Upon IFN-γ release they promote phagocyte activity, while upon CD40L expression can induce production of opsonizing and complement-fixing antibodies like IgM and IgG by B cells (16).

Th2 Lymphocytes

Th2 polarization from naïve CD4+ T cell is achieved thanks to the early production of IL-4 during primary response against helminths and allergens. These pathogens provide a persistent and chronic stimulation of T cells because of lack of inflammatory cytokines production and macrophages involvement (1). The early IL-4 production responsible for Th2 differentiation is provided by the naïve Th cell itself, upon Notch triggering by its ligand Jagged-1 expressed on Dendritic Cells (DCs) (17). Another thesis, instead,

support the hypothesis that the early IL-4 production should be attributed to mastcells, basophils and eosinophils. IL-4 intracellular pathway leads to STAT6 activation and, together with TCR antigen recognition, to GATA-3 expression. This transcription factor, strongly addresses to Th2 phenotype, triggering a positive feedback mechanism. Furthermore, GATA3 inhibits the IL-12β chain expression, thus avoiding IL-12 signal transduction and Th1 polarization. GATA3 binds to the main type 2 cytokines promoter, activating the transcription of a gene cluster that include IL 4, IL 5, IL 9, IL 13 and granulocyte/macrophage colony-stimulating factor (GM-CSF) genes. Th2 cytokines are important for the initiation, maintenance, and amplification of human allergic inflammation: IL-4 and IL-13 are involved in class-switching of B cells; IL 4, IL 9 and IL 13 in recruitment of mast cells, and IL 5 eosinophils in maturation and recruitment (1) (4).

Th17 Lymphocytes

Th17 cells and their effector cytokines mediate host defensive mechanisms to various infections, especially extracellular bacteria and fungi. The first evidence of this cell population date back to the study of Infante-Duarte (18) in which they demonstrated that naïve T cells, primed by lysate of B. burgdorferi, developed a phenotype with much higher IL-17A production than those from T cells primed under Th1 and Th2 conditions. In the earlier studies on mouse models, Th17 cells showed an heterogeneous cytokine production including IL-17A, IL-17F, IL-22, IL-26, together with IL-6 and TNF-α. These cytokines have different cell targets (fibroblast, epithelial cells and macrophages) and stimulate metalloproteases, nitric oxide synthases, chemokines and cytokines production, thus leading to a common output: recruiting neutrophils granulocytes and triggering a strong proinflammatory response. Human Th17 cells were later defined upon IL-17 production and ROR-γt, IL-23R, CCR6 e CCR4 expression (19) (20). RORγt together with STAT3 are important transcription factors for Th17 response. Unlikely mouse

Th17 cells, human studies do not provide univocal data regarding cytokines involved in Th17 development. The attention has focused on IL-1β, IL-6, IL-23 cytokines that are produced by DCs during fungi or bacterial infections (21).

Moreover, Th17 differentiation is restricted to CD4+ CD161+ T cell precursors, which are detectable in both human umbilical cord blood and thymus. Another cytokine has been reported to be important for the development of human Th17 lymphocytes: TGF-β. This cytokine does not have a direct effect on the genesis of human Th17 cells, but is able enhance Th17 polarization by suppressing T-bet expression and Th1 differentiation (22). Beyond their protective role in the clearance of extracellular pathogens, Th17 lymphocytes, are involved in the pathogenesis of many autoimmune diseases (23).

Regulatory T Lymphocytes

Another cell population belonging to CD4+ T subset, is the T regulatory (T reg) cell population. Characterized by the expression of high levels of IL2 receptor α chain (CD25) and FOXP3, a forkhead transcription factor, both crucial for genesis and maintenance of Treg phenotype. As expected for the low expression of CD127 and the high expression of CD25, T reg cells, unlike other T cell population, do not respond to IL-7, but mainly growth under IL-2 stimuli (1). We can make a distinction between Natural T regulatory cells (nTreg) and Induced regulatory T cells (iTreg) (4). The first ones originate in thymus from T CD4+ precursors upon self-antigen recognition, the second ones develop from naïve CD4+ lymphocytes in secondary lymphoid organs and do not display self-antigen specific TCR as they can also recognize non-self-antigens. TGF- β and IL-2 are essential for the Treg development, both in vitro and in vivo: TGF-β stimulate FOXP3 expression, while IL-2 signalling pathway leads to STAT5 activation that enhance FOXP3 levels as well. Treg carry out their immunosuppressive function by secreting inhibitory cytokines (TGF-β an IL-10) that down-regulate induction and proliferation of effector T cells (1).

Furthermore, they express CTLA-4 (cytotoxic T-lymphocyte associated molecule-4) and take direct contact with APCs blocking their stimulating function.

TGF- β exert many important functions in the immune system:

• Inhibits T cell proliferation and effector functions;

• Inhibits macrophage activations;

• Promotes IgA isotype switching, that is mainly involved in the mucosal immune response;

• Stimulates macrophages and fibroblast collagen synthesis, promoting extracellular matrix remodelling and tissue repair.

IL-10 is broadly produced by many cell types as DCs activated macrophages, but also by Th1 and Th2 cells. IL-10 acts with a negative feedback mechanism in those cells inhibiting IL-12 production and MHC class II expression. Unlike conventional T cells, regulatory T cells do not produce IL-2 and are therefore anergic at baseline (4).

CD8+ T Lymphocytes

The other arm of effective response is represented by cytotoxic T CD8+ lymphocytes (CTL or Tc1). This population play an important role in immunity to cancer cells or cells that are infected by intracellular pathogens. The TCR binds to the MHC class I/antigen complex, while the CD8 co-receptor stabilize the interaction binding to the constant portion of the class I MHC molecule. The LFA-1/ICAM-1 binding also reinforce the interaction between CTL and their target cells. CTL express also typical NK KIR family receptors that bind to MHC class I molecules independently from antigen presentation, thus limiting abnormal cytotoxic response against normal cells (24).

The principal mechanism used to destroy target cell is the release of cytosolic proteins contained in the cytoplasmic granules of CTL like Granzyme and

Perforin. CTL are characterized by the expression on the surface of FAS ligand which can bind to Fas receptor expressed on the target cell, leading to Fas-associated death domain (FADD) recruitment, effective caspases activation and cell death. After the great discovery of the Th population, Mosman et al. investigates the cytokine profiles of CTL and pointed out that a small subset, beyond the classical IFN-γ and TNF-α producing CTLs, produces IL-4 and IL-5 demonstrating that CD8+ T cells can secrete either Th1-like or Th2-like cytokine patterns, and contribute to bystander B cell activation. (25)

Tumor Immunology

Cancer is a tissue lesion that is functionally, nutritionally and evolutionarily different and independent, than the normal tissue from which is derived. A pathological condition of a neoplastic nature can be caused by mutations of genes that control cell growth, from genetic alterations that convert a proto-oncogene into an oncogene, from infections by oncogenic viruses. As a consequence of these events, the cell can no longer properly control its growth and differentiation.

Neoplasms can be divided into benign and malignant. Benign tumors have localized growth and are limited in time, are made up of cells that are the same or similar to those of the tissue from which they derive. Malignant tumors have a more rapid and progressive growth, are equipped with invasive activity against surrounding tissues, they can propagate at a distance in other organs through the bloodstream (metastases) and have more or less dramatic signs of cellular atypia relative to the organ from which they originate.

Malignant tumors are classified according to the embryonic origin of the tissue from which they derive:

- Carcinomas, derive from tissues of endodermal or ectodermal origin such as skin or epithelia;

- Leukemia and Lymphomas, originate from hematopoietic cells of the bone marrow (the first proliferate as single cells while the latter tend to grow as tumor masses);
- Sarcomas, derive from connective tissues of mesodermal origin (bone, adipose tissue and cartilage).

Studies on the relationship between immunity and cancer began more than one hundred years ago and continued alternately. In the '70s, Burnet and Thomas proposed the immunosurveillance theory, which laid the foundation for the study of the relationship between the immune system and tumor growth. According to Burnet, the tumor cells' specific neo-antigens would provoke an immunological reaction capable of eliminating the emerging neoplasm (26). Moreover, according to Thomas, the most advanced organisms should have mechanisms of protection against tumors, similar to those that mediate allogeneic rejection. The immune system, in particular T lymphocytes, would be able to constantly monitor host tissues from the emergence of transformed cells recognizing tumor-associated antigens (TAA) (27).

This theory was soon abandoned due to the discordant results as result of the use of imperfect immunodeficiency models. In the second half of the '90s it was then recovered, following experiments that demonstrated the importance of different components of the immune system, both innate and adaptive, in the control of the neoplastic transformation: in fact, it was observed the presence, inside or at the periphery of tumor masses, of an infiltrate consisting of: T lymphocytes, NK cells and macrophages. Furthermore, individuals with congenital or acquired immunodeficiencies had an increased incidence of some neoplastic diseases (28).

The immunosurveillance was conceived as a binary process, of complete or absent protection of the host, able to act only in the early stages of the onset of the tumor. In reality, the immune system seems to have a more complex role: on the one hand it contrasts the tumor development and on the other hand it

remodels the neoplasm during its progression. The immunological remodeling of the tumor takes place continuously, even if the greatest effects of this action are probably in the early stages of growth, when the tumor is not yet clinically detectable.

To describe the behavior of the immune system, a new definition has therefore been proposed, "Cancer Immunoediting" (29), comprising three main phases called the "Three *Es*" (30):

- **Elimination:** it can give complete destruction of the tumor and resolution of the process without progress towards the following phases. Corresponds to the antitumor immunosurveillance stage**;**

- **Equilibrium:** survived genetically unstable tumor cells may be either maintained chronically or immunologically sculpted by immune "editors", which allows the neoplastic cells to survive, accumulate further alterations at the DNA level and modulate the expression of TAAs. The pressure exerted by the immune system on the tumor in the equilibrium phase is sufficient to control the progression of the disease, but at the same time allows a selection of neoplastic cells able to survive the immune response;

- **Escape:** tumor variants that have acquired insensitivity to the elimination begin to expand, the immune system is no longer able to control tumor growth**.**
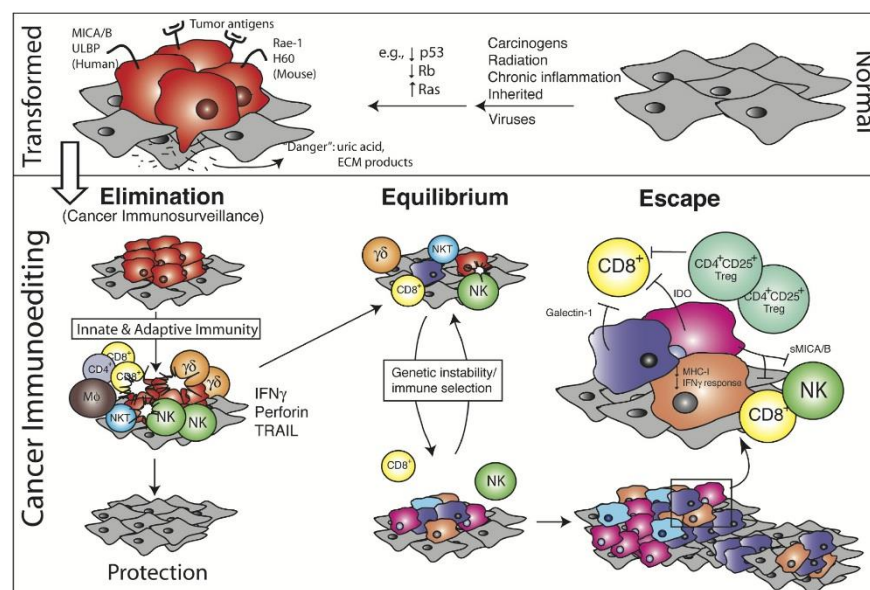

Figure 0: Cancer Immunoediting, the three Es (adapted from Dunn & al, 2004)

To assess how the immune system controls the development of tumors, several experiments have been performed on experimental models, altering specific components of the immune system and monitoring tumors development. Studies carried out by Shankaran have shown that RAG2 deficiency mice, spontaneously develope intestinal adenomas (50%), adenocarcinomas of the intestine (35%) and the lung (15%) at 15-16 months of age. Furthermore, when RAG2 deficiency was also associated with STAT1 deficiency (an important signal transducer for interferons) the incidence and the spectrum of the tumors were increased (31).

As said, the evasion phase begins when the balance between the immune system and the tumor moves in favour of neoplastic growth, due to the exhaustion of the immune response or its inhibition. The alterations of the tumor cells mainly concern the antigen processing and presentation mechanisms or the signalling pathway of the IFN-γ receptor. These alterations, reduce the recognition of tumor antigens and the consequent elimination of tumor cells ("tumor ignorance"). In addition, progressive selection also increases the ability of neoplastic cells to actively obstruct the protective functions of the immune system, producing inhibitory cytokines such as TGF-β or IL-10 (32), or other T-cell lymphocyte inhibitors, such as galectin-1 and the indoleamine 2,3-dioxygenase (IDO) enzyme (33).

Tumors can induce an altered differentiation of dendritic cells (DC) into more immature non-functional phenotypes (iDC). For example, in patients with different types of tumors, there was a noticeable decrease in DC and a prevalence of immature phenotypes, which expressed low or zero levels of costimulatory molecules (CD80, CD86). A small number of antigen-presenting cells (APC) makes immune stimulation ineffective and the presence of iDCs, which do not provide adequate T-cell costimulatory signal, may result in T-cell anergy. It has been proposed that several factors deriving from tumor, including VEGF, IL-10, IL-6 and M-CSF, constitutively activating STAT3 and

inhibiting NF-kB (nuclear factor kB), are responsible for this DC maturative block, that contributes to the suppression of immune responses (34).

Cytokines produced by tumors are also responsible for an altered hematopoiesis that lead to the generation of a population called Myeloid-Derived Suppressor Cells (MDSC). These cells, characterized in human by the expression of CD33, CD14 and low level of HLA-DR, are discriminated from other myeloid cell types in which they possess strong immunosuppressive activities, rather than immunostimulatory. During the neoplastic growth, the continuous release of cytokines, growth factors and chemokines stimulates the bone marrow hematopoiesis, generating a progressive accumulation of MDSC in the blood, in the spleen and in the tumor microenvironment.

MDSCs, besides reducing the already poor antitumor immune response, contribute with various mechanisms to neoplastic growth. First, the activation of enzyme arginase-1 (ARG1) in these cells metabolizes L-arginine, producing polyamines, which can contribute directly to the proliferation of tumor cells.

Moreover, MDSCs can directly damage the DNA of the surrounding cells, contributing to the neoplastic transformation, through the release of reactive and potentially mutagenic species such as nitric oxide, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (35) (36).

MDSCs are activated by T cells and cytokines produced by tumors, including VEGF, IL-1β and GMCSF; the mechanism by which CD4+ and CD8+ T lymphocytes are inhibited is complex, seems to be mainly associated with the activity of ARG1 and with the inducible nitric oxide synthase (iNOS or NOS2); both enzymes metabolize the amino acid L-arginine, giving rise to urea and L-ornithine or nitric oxide (NO) and L-citrulline. ARG1 (also known as hepatic arginase) causes a reduction of extracellular L-arginine, associated with the down-regulation of the CD3ζ chain of the T-cell receptor. Therefore, MDSCs induces T cell dysfunctions and promotes tumor growth through inflammation and angiogenesis (37).

The deprivation of amino acids is an important mechanism of regulation of the lymphocyte response, IDO is an important enzyme involved in the metabolism of tryptophan (Trp). It is produced by many cells, including cancer cells and APCs; the increase in IDO concentration follows a local depletion of tryptophan and an overproduction of its metabolites, which cause cell cycle arrest, anergy, T-cell apoptosis, increasing Treg activity (38) and (at list *in vitro*) can induce differentiation of naïve CD4+ T cells into Treg (39).

In this regard, some works that report the presence of Mesenchymal Stem cells (MSCs) in the stroma of some tumors appear particularly interesting (40). It is well established that bone marrow MSCs exert an *in vitro* immunomodulatory action on lymphocytes, reducing T cell proliferation and cytokine release (41).

Therefore, defining the possible role of these cells in the tumor stroma, can help us to better understand how the tumor microenvironment modifies the immune response and use these information to modify and develop new therapies for solid tumors.

Mesenchymal Stem Cells

Stem cells are defined as immature cell precursors endowed with self-renewal capability and with the great potential of multilinear differentiation. The work of Till and McCullouch (42) on mouse hematopoietic stem cells was undoubtedly the starting point for all subsequent research strategies on stem cells.

Through an asymmetric cell division called "bivalent mitosis", stem cell gives rise to two daughter cells. One identical to the stem cell itself, barely proliferating and able to maintain the pool of stem cells of the tissue unchanged, the other with proliferative capacity that progressively maturate towards phenotypically and functionally more specialized cells. With this asymmetric division the number of stem cells is kept unchanged, while the

most commissioned cells, will give rise to a significant number of mature cells that constitute the tissue (43).

Based on their differentiating potentialities, and therefore their plasticity, stem cells can be distinguished in (44):

▪ **Totipotent Stem Cells**: stem cells that can differentiate into all cells of the whole organism and even into cells of extra-embryonic tissues, such as the placenta. These cells derive from embryos at the stage of 4-8 cells, after 1-3 days from fertilization;

▪ **Pluripotent Stem Cells**: embryonic cells at the blastocyst stage, 4-14 days after fertilization. These cells are capable of giving rise to all the cells we find in an adult individual but not to cells that make up extra-embryonic tissues;

▪ **Multipotent Stem Cells**: cells that have the ability to replicate and stay in culture, but not to renew themselves in an unlimited way. They differentiate into different tissues, but belonging to the same embryo sheet. Adult stem cells belong to this category.

▪ **Unipotent Stem Cells**: present in adult tissues, more limited and organ specific, are able to self-renew and to differentiate in the cell type of the tissue they belong to, ensuring the repair and maintenance.

Mesenchymal stem cells (MSCs) are of particular interest, they are a non-hematopoietic precursor initially isolated from bone marrow as adherent, highly proliferating elements, with long-term self-renewal potential and multilinear differentiation in different tissues of mesenchymal origin (45) (46).

MSCs derive from the mesoderm, the intermediate embryonic leaflet from which the connective tissues of the whole organism originate, which differs around the third month of gestation. The mesenchyme differs greatly from the other embryonic sheets, as it is composed of an abundant extracellular matrix in which the mesenchymal cells are immersed. MSCs can also originate from

some portions of the other two embryonic sheets: neural crest ectoderm and the prechordal plate endoderm (47).

However, little is known about their development during fetal and post-natal life. It has been described the existence of a stromal support component, surrounding the primitive hemopoietic layer of the dorsal aorta in the aorto-gonad-mesonephric region of the human fetus. This fairly homogeneous cellular population consists of a layer of cells packed in roundish groups, located in the mesenchyme, expressing various proteins of the extracellular matrix, capable of supporting both embryonic and adult hematopoiesis (48).

A large proportion of cells analogous, for phenotype and cultural characteristics, to adult medullary MSCs, but with larger differentiation potentialities, was then found in the circulating blood of the human fetus up to the 7th week of gestation, after which, these cells begin to decrease by number, persisting until the 12th week (49). Experimental evidence indicates that a very low-frequency cell population persists in peripheral blood (50), colonies of fibroblast-like cells, expressing various mesenchymal characteristics, were obtained in culture in the presence of fetal bovine serum, from healthy subjects blood samples, without addition of growth factors (51).

It can therefore be assumed that, during embryo-fetal development there are cells that are distributed to the various body districts and persist in the adult as a reserve for tissue repair and regeneration (52).

MSCs are able to differentiate not only in tissues of mesenchymal origin, including marrow stroma, adipose tissue, bone, cartilage, tendon and skeletal muscle, visceral mesoderm and endothelial cells (46); but also in cells of non-mesodermal origin, such as neurons and (at least in mice) lung epithelial cells (53) (54).

Phenotypic characterization of MSCs is still an in-depth field given the lack of a specific marker for the analysis and isolation of MSCs (55). Currently, they are identified thanks to a combination of morphological, physical,

phenotypical and functional characteristics (56) (57): they have, in fact, the ability to grow in adhesion, appear tapered (similar to fibroblasts), and express on the cell membrane molecules of adhesion such as CD105, CD73, CD44, CD90, CD29 and STRO-1, while negative for leukocyte markers (CD45), hematopoietic stem cells (CD34 and CD133), express MHC I but not MHC II (58).

MSCs' role in Immunomodulation

Mesenchymal stem cells have attracted researchers and clinicians for their ability to regulate the immune response and leukocyte functions both *in vivo* and *in vitro* (59).

Different laboratory data show that MSCs are capable of inhibit T cells (60) (61), B cells (62), NK (63), DCs (64) (65) and also neutrophils (66).

Although regulatory mechanisms of MSCs on immune system are not fully understood, it is known that soluble factors and cell-cell contact-dependent mechanisms are involved in modulation of the immune system (67). They could exert their inhibitory role through different pathways, potentially inhibition of T cell may be due also to the suppression of activation, differentiation and maturation of DCs.

MSCs and DCs

MSCs inhibit differentiation, maturation, activation and function of DCs, by soluble factors (such as IL-6 and TGF-β) (68) and/or to cell-cell contact, resulting in generation of immature DCs. The inhibition of the DCs occurs in G0 / G1 phase (69). The inhibitory effect, seems to be different if it is mediated by cell-cell contact or by soluble factors. Many studies report the cell-cell contact as the major responsible of DCs inhibition (65), in particular NOTCH pathway (70).

Influence of MSCs also plays a role in DCs by reducing endocytosis capacity and inhibiting the production of IL-12 (65). Insufficient generation of IL-12 can

causes induction of anergic T cell and thus tolerance. Therefore, by the inhibitory action of MSCs on DCs, many immune functions could be inhibited.

MSCs and NK cells

MSCs prevent IL-2-induced NK cell proliferation and exert an inhibitory effect on these cells' functions such as cytotoxicity (63). The number of MSCs determine their effects on NK cells, at low NK-to-MSC ratios, MSCs alter the phenotype of NK cells and suppress proliferation, cytokine secretion, and cytotoxicity (71). It was reported that human MSCs inhibit NK cell-mediated cytolysis by nonclassical human leukocyte antigen class I (HLA-G) secretion (72).

MSCs and T lymphocytes

The inhibitory effects of MSCs on the proliferation of CD4+ and CD8+ T cells have been reported (60), MSCs inhibit the production of IFN-γ and increase the production of interleukin-4 (IL-4) by Th2. This situation changes T cells from pro-inflammatory (IFN-γ) state, to an anti-inflammatory (IL-4 production) state (61). Apart from impairing effect of MSCs on T-cells, they inhibit T-cell formation by silencing T-cells at G0/G1 phase of the cell cycle (73). MSCs reduce T-cells proliferation by the mechanism of cell-cell contact as well as soluble factors such as: NO, IDO, IL-6, IL-10 and TGF-β (60) (74).

The first step probably involves cell-cell contact and is mediated by adhesion molecules. For example, following direct interaction with T lymphocytes, MSC releases IL10, which plays a key role in the secretion by MSC of soluble HLA-G5, that suppress T cells activation, reduces cytotoxicity of CD8+ T cells and promotes the generation of Treg (72).

Soluble factors like NO or IDO, are produced by MSCs upon stimulation received by cytokines, like IFN-γ secreted by T or NK cells. In fact, IFN-γ is able, by binding to its receptor (CD119) on MSCs cells, to induce the expression of the IDO enzyme and to increase the secretion of some of the soluble factors

listed above, leading to an increased immunosuppression and consequent reduction of lymphocyte proliferation. The IDO inhibitory activity is mainly due to the depletion of tryptophan for the microenvironment and also to the production of catabolites kynurenine (75) (76). Other soluble factors, such as TGFβ1, HGF (hepatocyte growth factor), IL10, prostaglandin E2 (PGE2), HO1 (heme oxygenase 1), IL6 and HLA-G5 soluble, are constitutively produced by MSC and their secretion can be further increased by cytokines released from target cells, after interaction with MSCs (76) (77).

Head Neck Squamous Cell Carcinoma

Head and neck neoplasms represent a nosological entity whose importance is not univocally founded on clinical-prognostic aspects, but also on epidemiological ones. Indeed, HNSCC is the sixth among the most common malignancies worldwide and according to the American Cancer Society statistics, 5-year specific survival is around 65% (78).

Head and neck squamous cell carcinoma (HNSCC) comprise a heterogeneous group of tumors that arise from the squamous epithelium of the oral cavity, oropharynx, larynx and hypopharynx. The diversity of tissues present, determines the different frequencies of the different tumor histology, in turn characterized by biological and peculiar evolutionary mode behaviors. In fact, there are rare tumors that can originate from various tissues: the adenocarcinomas, that originate from salivary glandular epithelia or thyroid; melanomas from melanocytes and lymphomas from the lymphatic tissues. However, the most histological frequent and equally distributed in the various locations, is the squamous cell carcinoma, that developed by the epithelia lining the mucous membranes of the districts, and that is present in over 90% of cases (79). This type of neoplasia has a marked tendency to cervical lymphatic evolution and more rarely to blood metastasis.

The incidence of HNSCC is characterized by several factors; the geographic disposition is an example, HNSCC represents 50% of all tumors in the East-Asian continent, while in Europe and America it represents only 5%. It is also known that smoke and alcohol are the two major risk factors involved in the etiopathogenesis of neoplastic pathology, and especially when present in combination. However, approximately 5% of all oropharyngeal carcinomas occur in non-smokers or drinkers; this suggests that other risk factors may be responsible for the neoplastic transformation. One of the potential risk factors is papillomavirus (HPV) (80) (81), more than 100 types of HPV have been reported so far, and some of them have already been widely recognized to be able to support the neoplastic transformation of the squamocellular epithelium of the anogenital region, the uterine cervix, and the oropharynx (HPV 16 and 18) (82).

The survival of patients with carcinoma of the head and neck, even with the improvement of surgical techniques, complementary therapies (radio and/or chemotherapy) and integrated protocols now available, has remained substantially unchanged in the last 20 years: HNSCC has an high mortality rate.

Furthermore, it has been observed that the incidence of HNSCC increases in the context of acquired immunodeficiency; the patient's immune deficiency is associated with a worse prognosis, with an increase in recurrence and/or persistence of the disease. It is now well known, that patients with HNSCC have a severe immune deficit that affects the effector elements of the tumor defenses (cytotoxic T lymphocytes, NK cells, APC cells and macrophages) with consequent "tumor enhancement" mechanisms; probably, this condition of immunosuppression is due both to the immunosuppressive activity of the tumor and to the surgical and therapeutic treatments to which the patient undergo (83).

MSCs in HNSCC

Tumor stroma includes extracellular matrix and a heterogeneous population of cells that cooperate each other, such as myofibroblasts, mast cells, endothelial cells, tumor-associated macrophages and other leukocytes infiltrating the tumor, including T cells, NK and sometimes granulocytes (84).

All these cells, influence the process of carcinogenesis and tumor progression. Some studies discuss the hypothesis that tissue-resident and bone marrow MSCs migrate to the tumor and so they could represent the precursors of the stroma associated with HNSCC, promoting invasive growth and distant metastases (85).

In particular, the work carried out by Kansy in 2014, demonstrated for the first time the possibility to isolate, from HNSCC tumor sample, a population that met MSCs' criteria with the ability to support tumor growth (86).

Cytokines and growth factors secreted by the tumor, could recruit local and distant cells such as MSCs (87). Furthermore, an increase in serum's cytokines levels, is associated with HNSCC progression and relapse (88). Among these, PDGF-AB, PDGF-AA, VEGF, HGF and IL-8 exert a chemoattractant effect on bone marrow MSCs cells and are presumably involved in the recruitment of MSCs into the tumor (89). It follows that, the mobilization towards the tumor depends on the local and systemic inflammatory state. It is known that inflammatory cytokines stimulate the activity of specific metal-proteases of the matrix (MMP) in MSCs, allowing them to extravasate and migrate within the tissue (90).

In vitro and *in vivo* models, show that MSCs stimulate the invasive growth of solid and hematological tumors, in particular they support the neoplastic progression through the induction of lympho/angiogenesis, the modulation of the immune response and the generation of tumor associated myofibroblasts (89) (91) (92).

Angiogenesis is regulated by soluble factors produced by both tumor cells and stromal cells. VEGF and IL-8 are up-regulated in various solid tumors, including HNSCC, and are associated with invasive tumor growth and decreased survival (93). *In vitro,* VEGF acts on MSCs, leading to expression of markers typical of endothelial cells, such as VEGFR1, VEGFR2, VCAM-1, VE-cadherin and vWF (94). Furthermore, hypoxia (a typical condition in tumor environment), is a strong vascularization stimulus. In fact, it increases the MSCs' secretion of pro-angiogenic factors such as VEGF-A, FGF-2 (fibroblast growth factor), FGF-7, IL1, IL6, PDGF, TGFβ, TNFα (95) (96).

The involvement of regional lymph nodes is an important indicator of tumor aggressiveness and is a prognostic factor for HNSCC patients. The increase of tumor lymphatic genesis, correlates with lymph node metastases in HNSCC (97), in which VEGF-C seems to play an important role (98). Indeed, there is a direct correlation between the expression of VEGF-C and the presence of lymph node metastases in HNSCC (99). In patients with language carcinoma, VEGF-C associates with the size of the primary tumor, metastases to regional lymph nodes, distant metastases and prognosis. MSCs have an important role in lymphatic genesis and in the acquisition of a lymphatic phenotype when exposed to VEGF-C (100).

Myofibroblasts are abundantly present in the stroma of developing tumors and drive invasive tumor growth by providing a suitable environment (101). There is evidence of the pro-invasive growth activity of tumor-associated myofibroblasts (102). Myofibroblasts are fusiform cells with jagged nuclei with contractile capacity. The characterization of stromal myofibroblasts is based on a combination of markers for which they are positive such as α-SMA, γ-SMA, desmin and vimentin and markers for which they are negative as CD31, CD34 and cytokeratin. Myofibroblasts produce extracellular matrix components, remodeling enzymes, growth factors, cytokines and chemokines that together create an environment that promotes invasive tumor growth

(102). Abundant presence of myofibroblasts in the stroma is associated with several clinicopathological features of HNSCC, including lymph node metastasis, disease stage and regional recurrence. Tumor-associated myofibroblasts are thought to arise from several mobilized cell types including migratory neighbors, such as tissue-resident MSCs or tissue-resident fibroblasts, and distant invaders such as BM-derived MSCs (87).

Myofibroblast differentiation is regulated by growth factors, mainly of the TGF-β family, which are abundantly secreted by HNSCC (103). Interestingly, MSCs exposed to cancer cell-conditioned medium (CCCM), acquire a myofibroblast phenotype, characterized by an increased α-SMA expression and an increased ECM, protease, and growth factor production. Moreover, gene expression profiling reveals similarities between CCCM-exposed MSCs and stromal myofibroblasts (104). Most studies investigating carcinogenesis and the progression, invasion, metastasis, and angiogenesis of cancer have focused on alterations in cancer cells. Recently, interactions between cancer cells and the stroma have attracted considerable attention, and increasing evidence has accumulated on this. Several researchers have gradually clarified the origins, features, and roles of cancer-associated fibroblasts (CAFs), a major component of the cancer stroma. The so-called isolated fibroblast populations in these studies are likely to be multipotent MSCs, since MSCs can be isolated from a variety of oral tissues by explant culture (105). The interaction between cancer cells and the stroma could be a potential target for anti-cancer therapy, as well as the generation of CAFs by MSCs.

Results

TIL are enriched in Treg cells, but mainly produce pro-inflammatory IFN-γ and TNF-α cytokines.

We enrolled 9 patients affected by HPV-negative HNSCC (Table 1) and investigated the phenotype of tumor-infiltrating leukocytes. As shown in Figure 1A, we observed no differences in the frequency of CD3+ T cells, CD19+ B cells and CD16+ NK cells in the tumor specimen, if compared to peripheral blood (PB). Additionally, among CD3+ T cells, CD4+ T helper and CD8+ cytotoxic T cells showed similar frequencies (Fig. 1B).

Then, we investigated the presence of CD4+ and CD8+ T regulatory (Treg) cells, defined by the co-expression of CD25 (the high affinity subunit of the IL-2 receptor complex) and the transcription factor Foxp3. Our data showed a selective enrichment of both CD4+ and CD8+ Treg cells in the tumor specimen, if compared to PB (Fig. 1C)

Finally, we investigated the functional features of tumor infiltrating T cells. To this aim, we activated freshly recovered cells from PB and tumor tissue, evaluating their cytokine production profile by flow cytometry. As shown in Figure 1D and 1E, the majority of both CD4+ and CD8+ cells collected were characterized by IFN-γ and TNF-α production, suggesting the prevalence of cells with a type 1 phenotype with a high pro-inflammatory potential. Production of IL-4 and IL-17, typical of type 2 and type 3 cell phenotypes, respectively, were instead negligible (Fig. 1D and 1E).

These data suggest that the tumor microenvironment is enriched of cells with anti-inflammatory capacities, thus reducing the potential anti-tumor immune response.

Patient	Sex	Age	Tumor site	TNM	Tumor Vol (cc)
1	F	51	Larynx	T3N0M0	12,74
2	M	64	Tongue	T1N0M0	0,8736
3	M	66	Larynx	T3N2cM0	4,94
4	M	71	Larynx	T1N1aM0	0,52
5	F	65	Mouth	T4BN1M0	1,04
6	M	76	Mouth	T4BN0M0	22,23
7	M	64	Larynx	T2N0M0	0,0364
8	F	66	Tonsil	T2N2cM0	1,95
9	F	66	Larynx	T2N1M0	0,39

Table 1: Characteristics of the patients enrolled

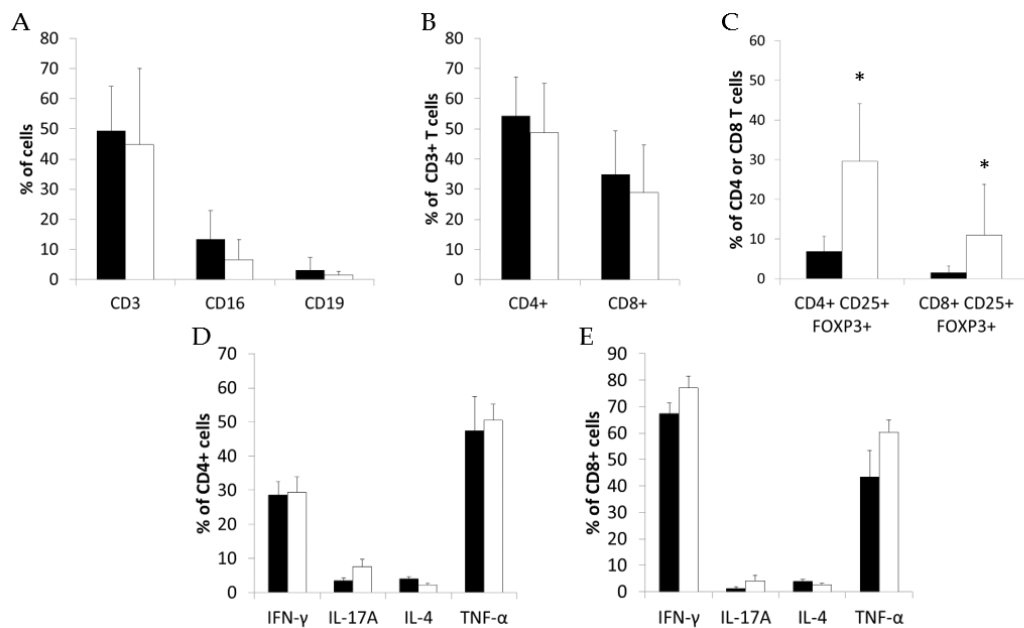*Figure 1: **(A)** Frequency of CD3$^+$, CD16$^+$ and CD19$^+$ lymphocytes in PB (black) and tumoral tissue (white) from 6 HNSCC patients. **(B)** Frequency of CD4$^+$ and CD8$^+$ cells among CD3$^+$ lymphocytes from patients. **(C)** Frequency of CD4$^+$ and CD8$^+$ Treg from HNSCC patients. Frequency of cytokine producing CD4$^+$ **(D)** and CD8$^+$ **(E)** cells from HNSCC patients. Columns represent mean+SEM. *p<0,05*

Pro-inflammatory IFN-γ and TNF-α increase MSC's immunosuppressive transcriptional signature.

We have already shown that HNSCC microenvironment is enriched in mesenchymal stem cells with immunosuppressive potential (40). Furthermore, it has been proposed that IFN-γ and TNF-α cytokines may affect the phenotype of bone-marrow derived mesenchymal stem cells (106). Thus, we decided to study how IFN-γ and TNF-α, the most produced cytokines by tumor infiltrating T lymphocytes, affect HNSCC-infiltrating mesenchymal stem cells. To this aim, we used five different frozen tumor-MSC lines, previously checked for their MSC characteristics (40). First, we examined if after thawing, tumor-MSC lines maintained their phenotype. To do so, we looked for the expression of markers as stated by the International Society for Cellular Therapy (56), comparing with MSC derived from Bone Marrow (BM).

As shown in Figure 2A, tumor- and BM-derived MSC express the same pattern of marker, thus indicating that the phenotype is maintained after thawing. Established that tumor-MSC lines were similar to those derived from BM, to investigate how IFN-γ and TNF-α affect tumor-MSC we obtained transcriptome data by microarray technology prior or after the exposure to the combined activity of IFN-γ and TNF-α. As a control, the same data were generated also from three BM-derived mesenchymal stem cell lines.

At first, we analysed the array data applying t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis, this to see clustering of our data. As shown in Figure 2B, our samples perfectly cluster in four different groups, thus indicating that our data were of high quality and consistent for the next analysis. Hierarchical clustering assisted heatmap analysis (Fig.2C) revealed that cytokine treatment significantly affects the transcriptional profile of both BM- and tumor-derived MSC. More importantly, cell lines from both sources could be distinguished either before or after cytokines treatment. Collectively,

these data show that HNSCC-derived MSC have a unique transcriptional signature, that is possibly the result of a close interaction with tumor cells, and that is retained even after cytokines stimulation.

Wondering if IFN-γ and TNF-α influence the same way the inhibitory activity of BM- and tumor-MSC, we checked our data looking for genes related to this activity and selectively induced by the two cytokines. As expected, based on the previous literature, *IDO1* was found among the most upregulated genes both in BM- and tumor-MSC lines. Furthermore, cytokines treatment significantly upregulated also transcript levels of the immunosuppressive molecules, *CD274 (PD-L1)* and *Interleukin 4 Induced 1 (IL4I1)* both in bone marrow- and in HNSCC-derived MSC lines (Fig. 2 D).
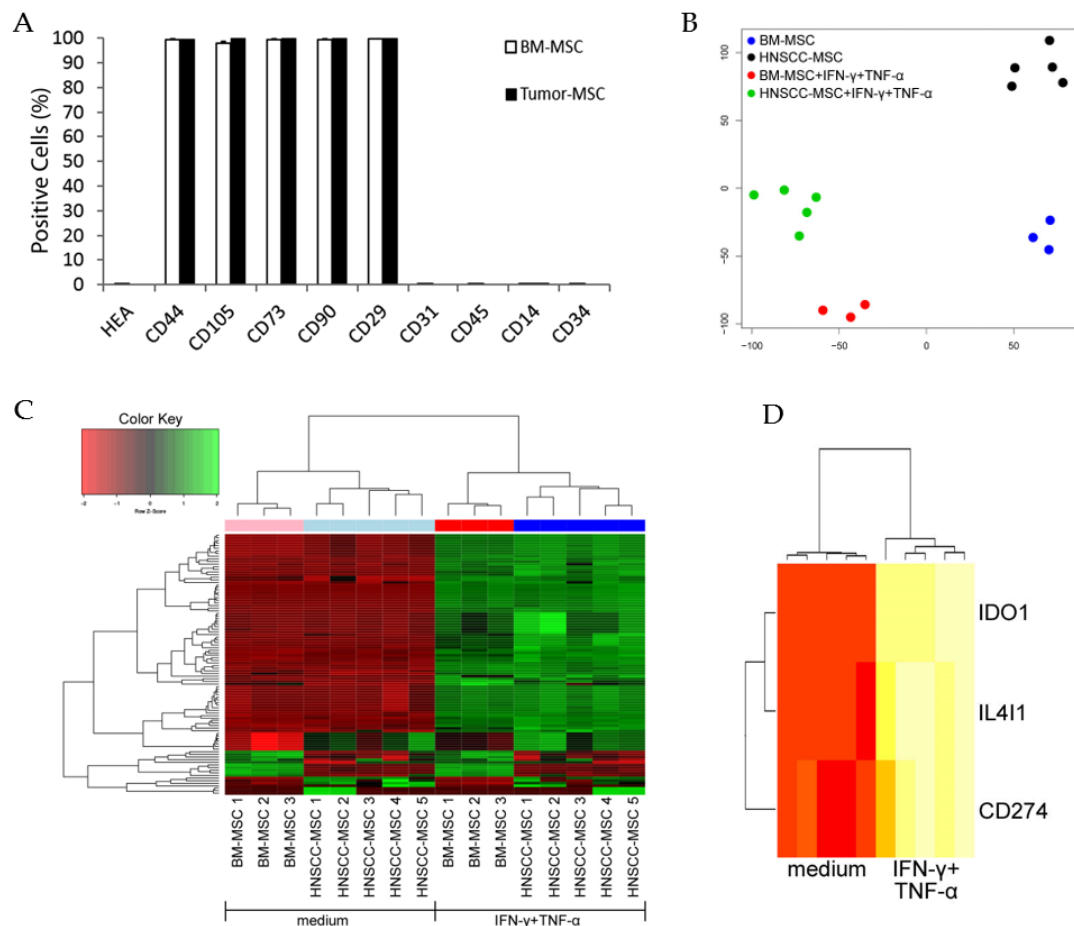

Figure 2: (A) Immunophenotyping of BM- and HNSCC-derived MSC. Columns represent mean+SEM. (B) t-SNE analysis performed on the microarray data. (C) Hierarchical clustering assisted heatmap analysis performed on transcriptome data generated with microarray technology on BM- and HNSCC-MSC resting (medium) or following cytokine activation (IFN-γ+TNF-α). (D) Transcriptome data of immunosuppressive genes IDO1, IL4I1 and CD274 by resting or cytokine activated HNSCC-MSC.

IL4I1 and PD-L1 are involved in HNSCC-derived MSC immunosuppression.

We decided to confirm the expression of immunosuppressive molecules observed at transcriptome level. For this reason, we stimulated three HNSCC-derived MSC lines with IFN-γ and/or TNF-α and then we investigated IDO, IL4I1 and PD-L1 expression at mRNA or protein level. Regarding *IDO1*, qPCR data showed that resting cells do not express this gene (Fig. 3A). IFN-γ alone can significantly promote its expression, but the addition in culture of TNF-α can further upregulate its transcription. TNF-α alone instead exhibited no activity on *IDO1* expression. Regarding *IL4I1*, again we observed no transcription by resting cells. IFN-γ and TNF-α alone exhibited only a mild effect on *IL4I1* transcription but when added together in culture allowed maximal gene expression (Fig. 3B). Lastly, we analyzed PD-L1 protein expression by flow cytometry. Resting cells showed basal PD-L1 levels, that were increased by both cytokines when used alone, even if with a prominent effect by IFN-γ. However, we observed again a synergistic effect of the two cytokines when used in combination, which allowed maximum membrane expression of PD-L1 (Fig. 3C).

Finally, we functionally tested the contribution of these three molecules to HNSCC-derived MSC immunosuppression. We activated, with anti-CD3 plus anti-CD28 antibody, in vitro CD4+ T cells freshly sorted from peripheral blood of healthy donors. All this, in presence of scalar doses of HNSCC-MSC and of selective inhibitors of IDO, IL4I1 and PD-L1.

As shown in Figure 3D, HNSCC-MSC alone were capable of inhibiting T cell proliferation in a dose-dependent manner. The addition in culture of L-methyltryptophan, an inhibitor of IDO, completely restored T cell proliferation, even at low HNSCC-MSC/T cells ratio. We blocked the PD1-PD-L1 pathway using an anti-PD1 neutralizing antibody, and the IL4I1-mediated

H$_2$O$_2$ production, via the addition in culture of the enzyme catalase. Inhibition of IL4I1 activity or PD-L1 pathway led to a significant restoration of T cell growth, despite being far away from the proliferation levels achieved with the addition of L-methyltryptophan. Collectively, these data indicate that, despite at lower levels if compered to IDO activity, PD-L1 and IL4I1 contribute to HNSCC-derived MSC immunosuppression activity. In addition, it must be noted that IL4I1-mediated H$_2$O$_2$ production, is achieved not only via phenylalanine metabolism but also via tryptophan degradation. Thus, addition in culture of L-methyltryptophan partially affects also IL4I1 activity, and T cell proliferation may benefit from the contemporary inhibition of two immunosuppressive mechanisms.
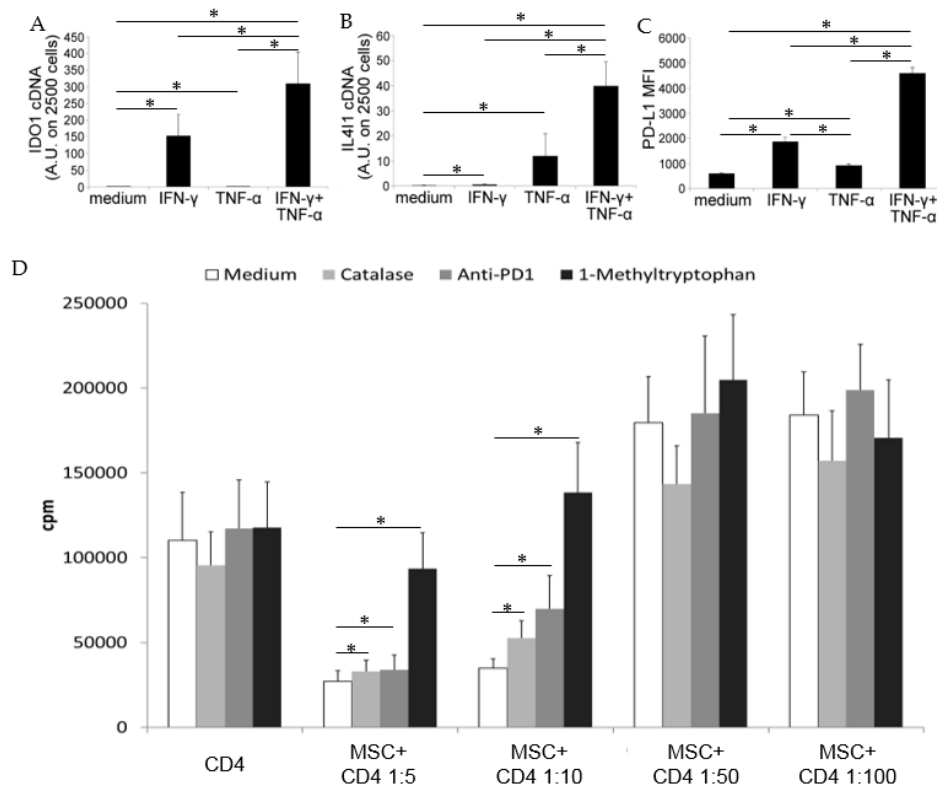

Figure 3: Evaluation of IDO1 (A) and IL4I1 (B) mRNA expression by HSNCC-MSC following single or combined stimulation with IFN-γ and TNF-α. Columns represent means+SEM of 5 independent experiments performed. *p<0,05. (C) Flow cytometric evaluation of PD-L1 protein expression. Columns represent means of fluorescence intensity +SEM. 5 independent experiments. (D) CD4+ T cells proliferation was assessed by 3H TdR incorporation following 3 days co-colture with different doses of HNSCC-MSC. Catalase (inhibitor of IL4I1 activity), neutralizing anti-PD1 mAb or 1-methyltryptophan (inhibitor of IDO1 activity) were eventually added to the cultures. Columns represent means+SEM of 7 independent experiments performed. *p<0,05.

IFN-γ and TNF-α increase MSC's chemokines production and lymphocytes adhesion.

Our data show that HNSCC-MSC exert an immunosuppressive activity on T cells in a dose dependent manner. This finding suggests that, despite some of the previous inhibitory mechanisms are not cell-contact dependent, they require that HNSCC-MSC and target T cells are in the same microenvironment. In line with this, we have already shown that both BM-MSC and HNSCC-MSC can produce the T cell recruiting chemokine CXCL10 upon IFN-γ and TNF-α stimulation (40). However, in that paper, we observed that conditioned media from both BM- and HNSCC-derived MSC were even more powerful than recombinant CXCL10 to favor T cell migration, thus suggesting that additional chemotactic factors may be released.

Taking advantage of our transcriptome data, we investigated the presence of chemokine genes upregulated by IFN-γ and TNF-α stimulation. Indeed, we found that cytokine stimulation could induce the expression of a plethora of chemokine genes such as CXCL9, CXCL10, CXCL11, CCL2, CXCL5, CCL5, CXCL2, CXCL1, CXCL8, CCL7, CCL13, CCL8 and CCL3, thus exponentially amplifying their leukocyte attraction capacity (Fig. 4A). Indeed, has mentioned above, MSC can exert their inhibitory role not only on T cells but also on neutrophils, macrophages, NK cells and DCs (107).

Further investigating the mechanisms of cellular contact, we wondered if the inflammatory cytokines also increased the expression of adhesion molecules that could then, together with the chemoattractant action, capture the attracted lymphocytes.

Checking our array data, we found an increase in Vascular Cell Adhesion Protein 1 (VCAM-1 or CD106), a protein usually expressed by endothelial cells after inflammatory cytokines stimulation. As shown in Figure 4B, we confirmed by flowcytometry, the increased expression of CD106 on MSC after

the stimulation with cytokines. In particular, we noticed that IFN-γ has a mild effect, while TNF-α play a major role on CD106 expression, of course the synergistic effect of the two cytokines lead to an higher upregulation of the protein.

To conclude, we functionally tested the ability of MSC to bind T lymphocytes in a co-culture experiment. Actually, after treatment with IFN-γ and TNF-α, MSCs were capable to bind T lymphocytes (Fig. 4C). This ability is due, at least in part, to CD106 expression as demonstrated by the reduction of the number of adherent T cells when an anti-CD106 antibody was added in the culture (Fig. 4D).
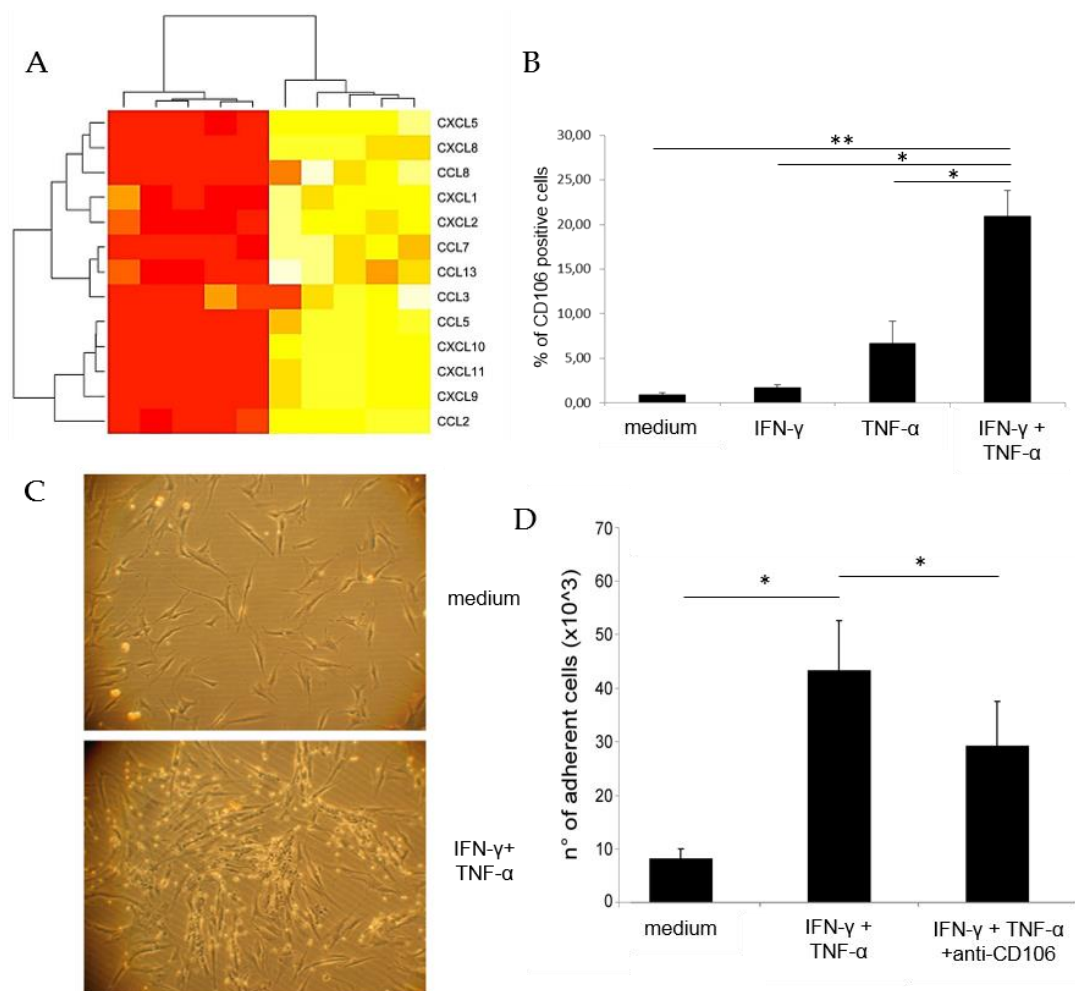
Figure 4: (A) Transcriptome data of chemokines by HNSCC-MSCs prior or after the treatment with IFN-γ and TNF-α. (B) Flow-cytometric evaluation of CD106 protein expression. Columns represent means+SEM of 5 different experiment performed. *p<0,05; **p<0.01 (C) Photography of HNSCC-MSCs and bounded T cells, with or without the treatment with IFN-γ and TNF-α, adherent T cells can be identified as the bright dots. (D) Numeric evaluation of adherent T cells, calculated by the MACSQuant flow cytometer. Columns represent means+SEM of 5 different experiments performed. *p<0,05.

CD4+ T lymphocytes inhibition, is not dependent on MSCs contact.

Given the ability of MSCs to attract and bind T lymphocytes, we wondered if cellular contact played an important role in inhibitory action. As regards CD4+ T cell proliferation, it has been demonstrated in literature (59) and, since IDO is the most involved factor, we can deduce that this is not related to cellular contact. We then asked ourselves if other T cell's characteristics were inhibited and how.

To this end, we adopted a Transwell® co-culture system, with small pores that did not allow T cells to migrate and come into contact with MSCs. After five days of culture, we recovered T cells and we checked their phenotype, looking at the expression of activation markers and also at cytokines production.

As shown in Figure 5A, when activated by the presence of anti-CD3 plus anti-CD28 mAb, CD4+ T cells upregulate, as expected, many markers such as CD69, CD25, CD154 (CD40L), CTLA-4, PD1 and downregulate IL7R (CD127).

When in the same condition we added MSCs, both in classic co-culture or in Transwell, we observed a completely opposite behavior. In fact, CD4+ T cells cultured in presence of MSCs, lose their ability to be activated, as demonstrated by the significant reduction in the expression of activation markers and the stability in the expression of CD127 (Fig.5A). The only exception is the expression of CD69, which is upregulated even in presence of MSCs without any further signal on CD3 or CD28. Moreover, its expression seems to be fortified by cell contact (Fig.5A). In any other cases, there are no differences between the two different culture condition.

Regarding the ability of CD4+ T cells to produce cytokines, we evaluated this after polyclonal stimulation with PMA/Ionomycin. As shown in Figure 5B, also the production of different type of cytokines is compromised by the inhibition due to MSCs. Indeed, T cells when cultured in presence of MSCs,

even if activated with anti-CD3/28, show a clear reduction of cytokines production ability, without significant differences between the two different culture systems.

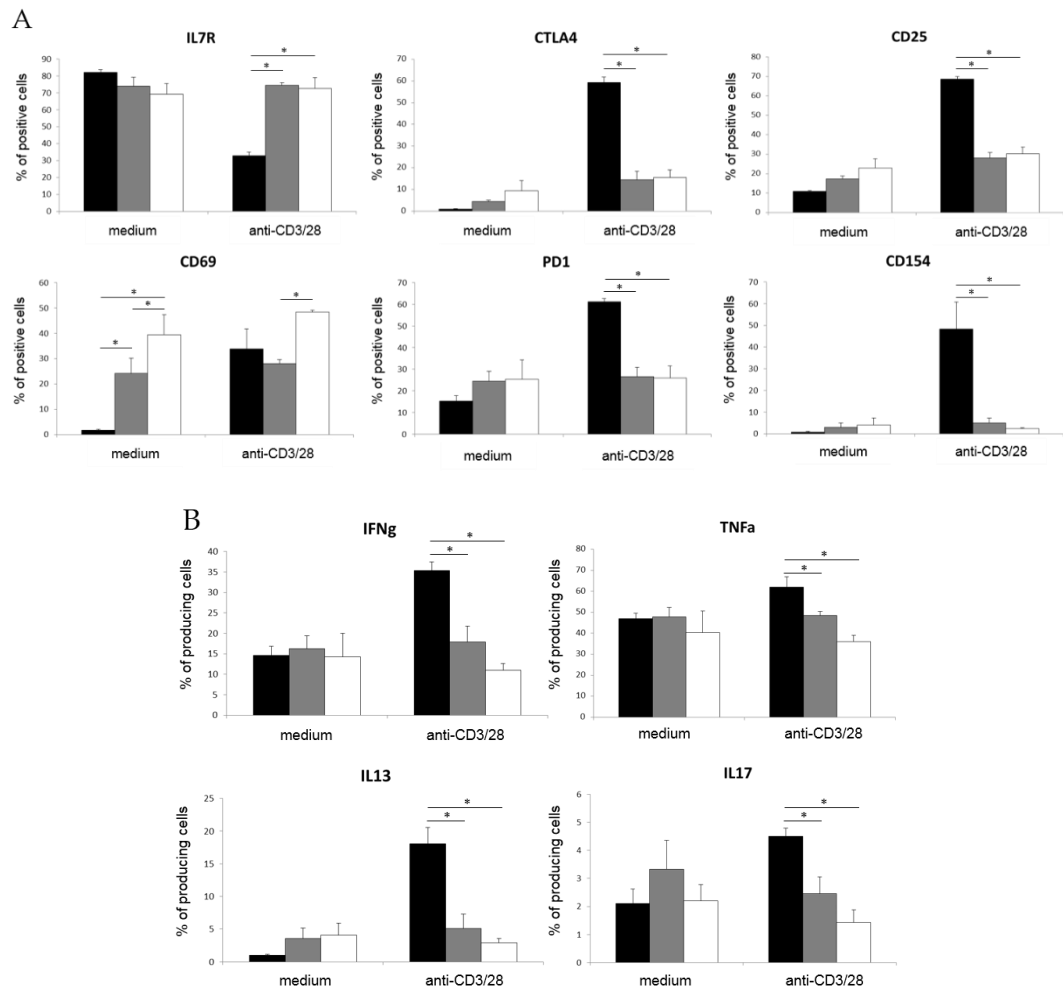*Figure 5: (A) Evaluation of different activation markers expressed by CD4+ T cells alone (black), with MSCs in a transwell plate (grey) and in a classic co-culture (white). (B) Evaluation of cytokines production by CD4+ T cells in the same conditions as above. Columns represent means+SEM of 3 independent experiments performed. *p<0,05*

Discussion

The last decades of immuno-oncology research, have provided solid evidence that tumors are recognized by the immune system and their development can be stopped, or controlled for long term, through a process known as immunosurveillance. In many cancers, however, malignant progression is accompanied by profound immune suppression that interferes with an effective antitumor response and tumor elimination. Initially, the escape from immunosurveillance was ascribed to changes in tumor cells themselves (i.e. loss of tumor antigens), making them a poor target of an immune attack.

However, it has become clear that immunosurveillance escape comes also from the ability of tumors to subvert normal immune regulation to their advantage. The tumor microenvironment can promote accumulation of immunosuppressive cell populations (108). The best described are regulatory T cells (Treg), myeloid-derived suppressor cells and mesenchymal stem cells (MSC).

MSCs are essential components of the tumor stroma, and play a central role in the tumor microenvironment, modulating growth and development of the tumor. Such cells are most likely responsible for the generation of cancer-associated fibroblasts (CAFs) (87), which contribute to the expansion of the fibrovascular network and tumor progression. MSCs are also able to act as precursors of endothelial cells and pericytes, and to promote angiogenesis and lymphogenesis through the production of soluble factors. Generally, MSCs are believed to reach tumor from the bone marrow (85), as strong evidence shows that MSCs migrate to the site of damage in various pathological conditions, including inflammation, tissue repair and neoplasms. During the progression and development of tumors, a large number of MSCs can be recruited at the tumor site. Furthermore, it is well known that BM-MSCs possess immunoregulatory properties on various cells of the immune system, in both in vitro and in vivo models (67).

We have recently shown that HNSCC-associated MSC can modulate the anti-tumor immune-response, favoring tumor growth (40). In this study, we wanted to investigate more about the mechanisms involved in the immunosuppressive activity of tumor-MSCs.

First of all, we observed that Tumor Infiltrating Lymphocytes in our HNSCC samples, were significantly enriched for T regulatory cells, thus indicating a potential reduction in anti-tumor immune response. Nevertheless, most of the effector T cells were characterized by IFN-γ and TNF-α production, therefore, with high pro-inflammatory potential.

Then we wondered how the tumor pro-inflammatory environment affects infiltrating MSCs. Our data clearly show that HNSCC-derived MSC have a unique transcriptional signature and cytokine treatment significantly affects the transcriptional profile of both BM- and tumor-derived MSC.

Since we wanted to investigate the immunosuppressive activity of tumor-MSC, we looked for genes related to this pathway and selectively induced by the two cytokines. We found out that, besides the genes already known to be expressed by MSC upon inflammatory stimulation, as IDO1, also other immunosuppressive genes were up-regulated, like IL4I1 and PD-L1.

Regarding PD-L1 (CD274), it's a transmembrane protein that play a major role in suppressing the immune system. Several human cancer cells expressed high levels of PD-L1, and its blockade reduced the growth of tumors in the presence of immune cells. Its binding to PD1 (expressed on lymphocytes), leads to a reduction in T cells proliferation. It has been proved that IFN-γ and TNF-α up-regulate PD-L1 on BM-MSCs (106), but it's the first time that this is demonstrated on tumor-derived MSCs.

Instead, regarding IL4I1, it's an L-phenylalanine oxidase expressed by tumor associated macrophages, mature dendritic cells, iTreg cells and Th17 cells, and it exerts an immunosuppressive activity by inducing CD3ζ chain downregulation through the enzymatic production of H_2O_2 (109).

We performed functional test to evaluate the contribution of this molecule on CD4+ T cell inhibition. The results show that, in our culture conditions, IDO was the major player in MSCs' mediated immunosuppression. Despite that, also IL4I1 and PD-L1 inhibition led to a slight increase in T cell proliferation. However, it must be taken into account that, since the strength showed by IDO activity, the elimination of tryptophan from the microenvironment could not allow T cells to reach high proliferation levels, even in presence of the other inhibitors.

Another important data showed by the microarray analysis, is the abundance of chemokines produced by HNSCC-MSCs upon IFN-γ and TNF-α stimulation, suggesting their high potential to attract leukocytes. Of note that among all the chemokines produced by MSCs, none seems to be related with Th2 recruitment. Thus, could be interesting to evaluate if MSCs have the ability to attract only a specific subset of T cells. Moreover, this could explain why it is believed that a Th2 signature correlate with bad prognosis. In fact, could it be that MSCs activated by the tumor pro-inflammatory environment, attract and inhibit only Th1, leading to a relative increase of infiltrating Th2.

Furthermore, we found out that HNSCC-MSCs, under inflammatory conditions, were able to express CD106 and this led to T lymphocytes binding.

The ability of our tumor-derived MSCs to express CD106, increase the reliability in defining such cells as "mesenchymal stem cells". Indeed, it has been demonstrated that fibroblasts do not upregulate CD106 expression in the presence of TNF-α (110).

It is also interesting to notice that Kansy & al (86) showed that MSC derived from tumor sample are able to produce inflammatory cytokines like IL-6, IL-8, IFN-γ and TNF-α and also adhesion molecules such as VCMA-1 (CD106) and ICAM-1 (CD54). These data indicate a curious and bi-directional role of MSCs, in fact, on one hand MSCs seem to have the ability to attract, bind and activate lymphocytes (by the cytokines release), but on the other hand MSCs

inhibit them and promote the growth of cancer cells, as showed by the co-injection of tumor cells and MSCs in muse xenograft model (86) (111).

In our settings, CD106 expression and T cells adhesion, are not responsible for the inhibition of T cells (as demonstrated by the transwell experiments), the only appreciable effect is the increase of CD69 expression, that is related with cell contact.

This interesting upregulation mediated by HNSCC-MSCs, could be related to CD69 role in Trp uptake (112). Since MSCs led to high deprivation of Trp amino acid from the microenvironment, it is likely that T cells respond upregulating CD69 to increase Trp uptake. Furthermore, CD69 is also a typical marker of tissue resident memory T cells, that promote their retention into the tissue (113). Thus, indicating a possible action of MSCs in lymphocytes recruitment and retention into the tumor.

Taken together, these data introduce an interesting hypothesis, namely that HNSCC-MSCs mirror BM-MSC in the bone marrow niche. Here indeed, BM-MSCs are able to bind (through CD106) lymphocytes (114), that are maintained as resting cells and their survival is conditional on IL-7 receptor signaling (115).

In fact, from an initial analysis, HNSCC-MSCs are able to produce IL-7 and induce an increase in S6 ribosomal protein phosphorylation (data not shown), indicating an activation of mTOR pathway. This fact is particularly curious, since proper mTOR activation is entirely dependent on amino acid presence in the environment. Therefore, low amino acid availability potently inhibits mTOR activity, that it is strictly related with T cells activity. Hence, fine tuning mTOR signaling, through amino acid uptake and intracellular signaling pathways, can determine the phenotypic outcome and plasticity of activated T cells (116).

It will be interesting to further investigate this hypothesis. It could be also interesting to study if this hypothetical mechanism is present in non-

pathological tissue, and if it could represent the way in which Tissue Resident Memory (Trm) T cells are maintained in the tissue. In this scenario, it will be fascinating to find in which way T cells are re-activated when they need to respond to pathogens and in this case, use this mechanism to re-activate T cells against tumor.

Moreover, a recent study (111) showed that BM-MSC are able to increase PI3K/mTOR activation in tumor cells, thus leading to an higher proliferation rate and inhibition of apoptosis. They associate this effect to the increase of Periostin secreted by tumor cells themselves. However, in the study, they do not delve into the mechanisms that lead to this increase. It will be interesting to evaluate if this effect on tumor cells is mediated by the same mechanisms, or similar, that induce and increase of mTOR pathway on T cells.

The data of this thesis are interesting above all given the new therapies adopted, or in the course of clinical trials, in the cases of HNSCC. Indeed, in recent years many different clinical trials were started to prove the efficacy of some checkpoint inhibitor, like anti-PD1/PD-L1 (117). Prior to the advent of these immune checkpoint inhibitors, there had been limited advances in the systemic therapy of recurrent or metastatic HNSCC.

There is currently an intense international research effort investigating immune combination therapy where novel immunomodulatory agents are combined with an anti-PD1/PD-L1 backbone. These, include combinations of PD1/PD-L1 inhibitors with agent targeting Indoleamine 2,3-dioxygenase 1 (IDO1), Epacadostat (118). This combination showed good response rate and favorable toxicity.

Could be interesting to study tumor-associated MSCs and tumor infiltrating lymphocytes on patients participating in clinical trials involving PD-L1 and IDO1 inhibitor. Moreover, could be intriguing to further investigate the hypothesis of MSCs as "*feeder cells*" for Trm, in the tumor context as in physiological context.

To conclude, our data together with the previous ones we produced (40) (the observation that the frequency of tumor-MSCs directly correlates with the size of the tumor and inversely correlates with the number of the TILs), consolidate the hypothesis that tumor-MSCs could promote tumor growth, directly or indirectly inhibiting the immune response.

Material and Methods

Patients Recruitment

Nine patients with Head-Neck Squamous Cell Carcinoma (HNSCC), confirmed by the respective biopsies, were enrolled with the consent of the University Hospital of Florence. All participants signed an informed consent and the procedures followed in the study were approved by the ethics committee of the Careggi University Hospital (AOUC). The site and the tumor stage were classified according to the TNM classification AJCC, 2010 (119).

The volume of the primary tumor was calculated based on diagnostic images. Tumor samples were obtained under general anesthesia during the surgical procedure, under sterile conditions. The peripheral blood samples were taken when the patient arrived in surgery. Inclusion criteria for patient enrollment in the study were:

1) patients with HNSCC (confirmed by biopsies) HPV-negative,

2) patients with primary head-neck tumor, not previously treated,

3) patients who never had cancer,

4) absence of systemic metastases.

A computerized tomography (CT) check was performed one month after surgery and twice more in the year following it, and/or after radiation therapy, in accordance with the ethical criteria of the Regional Committee on Human Experimentation.

A description of the patients is summarized in Table 1.

Reagents and Instruments

The medium used for lymphocytes cultures, was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2 x10^{-5} M 2-mercaptoethanol (2-ME) (all from Gibco Laboratories, USA), and 10% Fetal Bovine Serum (HyClone™, GE Healthcare,

USA). The culture medium used for mesenchymal cells was DMEM (Dulbecco's Modified Eagle Medium) high glucose (from Euroclone, Italy), supplement with 4 mM L-glutamine and 15% HyClone. Trypsin / EDTA used to detach adherent cells, was purchased from Euroclone.

Density gradient stratification was performed on Ficoll Lymphoprep™ of NycomedPharm (Norway). CD4 Isolation Kit II Miltenyi Biotec (Germany) was used for the purification of CD4 + T lymphocytes from peripheral blood samples. For the immunomagnetic separation, a VarioMACS type magnet was used with CS columns (Miltenyi Biotec).

Monoclonal antibodies used for cytofluorimetric analysis were purchased from BD Biosciences (USA), with the exception of the anti-CD106 antibody (V-CAM-1, Southern Biotech, USA), of the anti-CD29, anti-CD105 (Ancell Corporation, USA) and of the anti-PD1 (Biolegend, USA). Phorbol 12-myristate 13-acetate (PMA), Ionomycin, Brefeldin A, Formaldehyde, Saponin and Bovine Serum Albumin (BSA) were from Sigma Aldrich Co. (USA). PBS buffer, pH 7.2 without Ca^{2+} and Mg^{2+}, was from Euroclone.

RNeasy Micro Kit, from Qiagen (Germany) was used for mRNA extraction. TaqMan® Reverse Transcription Kit was from Applied Biosystem (USA) and the thermocycler "Master Cycler" used to perform RT was from Eppendorf (Germany). Real Time PCR was performed using an ABI PRISM® 7900HT Sequence Detection System from Applied Biosystems. The flow cytometer used for sample analysis was a BD LSRII, with FacsDIVA software (BD Biosciences).

Tissue samples

Tumoral samples, obtained at the same day of surgery, was digested with 1 mg/ml of Collagenase A (Sigma Aldrich), shaking at 37°C for 90 min and then filtered through a 40μm cell strainer (Corning, USA) to obtain single cell suspension.

The monocellular suspension thus obtained was subjected to both immunophenotypic analysis by flowcytometry and cultured in plastic flasks in DMEM with 15% FCS, in order to select mesenchymal stromal cells. The cultures were incubated at 37° C in a 5% CO_2 atmosphere. After 72 hours of culture the non-adherent cells were removed. When adherent cells reached a confluence of about 70-80%, they were detached by the use of Trypsin/EDTA solution (0,25 mg\ml at 37° C for 5 minutes), collected and expanded into larger flasks. Normally a homogeneous cell population is obtained after about 3 weeks of culture. For our experiments, we used MSCs lines obtained as described above and then frozen and stored in liquid nitrogen.

Cells recovery

Peripheral Blood Mononuclear cells (PBMCs) suspensions were obtained by centrifugation (1500g for 20') on Lymphoprep™ gradient (Oslo, Norway) of Buffy Coat of healthy donors provided by Meyer Hospital's transfusional center. After two wash steps with PBS, PBMCs were counted using a Neubauer chamber.

Immunomagnetic separation of CD4 + T lymphocytes

In order to obtain the CD4 + T population, purification by negative selection was carried out with CD4 isolation kit II. Briefly, PBMCs were incubated for 5 minutes at 4° C with the cocktail of biotinylated antibodies present in the Kit. This cocktail contains the following biotinylated antibodies: anti-CD8, -CD14, -CD16, -CD19, -CD34, -CD56, -CD123, -TCRγδ and Glicophorin A. After the incubation time, anti-biotin antibodies conjugated to superparamagnetic microbeads were added. The cells were incubated for another 10 minutes at 4° C, and then washed with 0.5% PBS pH 7.2+ FCS and resuspended in the same buffer. Then the cell suspension was loaded onto a MACS® CS Column, which is placed in the magnetic field of a VarioMACS Separator.

Flow cytometric analysis

Cell surface staining

Cells were washed in PBS+0.5% BSA and centrifuged at 300 g for 5 minutes. Subsequently, 5µl of rabbit γ-globulins (10mg/ml) were added directly to the cell pellet in order to saturate non-specific binding site (i.e. Fc-Ig receptors). Cells were stained with fluorochrome conjugated mAbs cocktail and incubated for 15' on ice, or at RT if CD127 was present. After the staining, cells were washed with PBS+0.5% BSA, centrifuged at 300 g for 5' and resuspended in 500µl of the same buffer. Samples were analysed by flow cytometry. Dead cells were discarded during the acquisition process by the use of Propidium Iodide (PI) provided by Invitrogen, at a concentration of 2 µg/ml.

Cytokines intracellular staining

Cells were stimulated with PMA/Ionomycin (10 ng/ml and 1 µM, respectively) for 6 hours (the last 4 hours in the presence of Brefeldin A, a Golgi inhibitor, 5µg/ml), washed with PBS, centrifuged at 300 g 5' and then fixed in formaldehyde 2% (15' at RT). After fixation, cells were washed with PBS+0.5% BSA (300 g 5') and stained for both surface markers and cytokines with fluorochrome-conjugated mAbs, in a buffer containing 0.5% of Saponin that allows the mAbs to entry into cells. After the staining, cells were washed with the same buffer containing Saponin, in order to remove excess mAbs.

Microarray

Gene expression profiles on BM- or HNSCC- derived cell lines were assessed by cDNA microarray technique using the SurePrint G3 Human GE v2 8x60K Microarray (Agilent Technologies). Cells were stimulated over night with TNF-α (10ng/ml) and IFN-γ (2,5ng/ml). Unstimulated control cells were also prepared in absence of cytokines. Total RNA was extracted using RNeasy Micro Kit (QIAGEN) following manufacturer's recommendations, quantified using a NanoDrop-1000 spectrophotometer and quality-checked with an

Agilent 2100 Bioanalyzer. Total RNA was labelled with the One Color (Cyanine 3-CTP) Low Input Quick Amp Labeling Kit (Agilent Technologies) according to manufacturer protocol and hybridized to the microarray chips according to specifications in One-Color Microarray-Based Gene Expression Analysis Protocol. Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2565CA) using one color scan setting for 8x60K array slides (scan resolution 2 um/pixel). Imaging data was extracted using Agilent Feature Extraction Software version 10.7.3.1 with default parameters of protocol GE1_107_Sep09. Data preprocessing was performed using R/Bioconductor (version 3.6) Agi4x44PreProcess (version 1.22) package. Briefly, raw Feature Extractor data was loaded into R and further background corrected (method="half", offset=50), normalized (method="quantile") and filtered (wellaboveBG=60, isfound=60). Differential expression (DE) analysis were performed with limma package (version 3.34.6) considering DE signals with abs(logFC)>1 and adjusted (Benjamini-Hochberg) p-value < 0.05.

RNA isolation, cDNA synthesis and real-time quantitative RT-PCR.

Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Cells were lysed with RLT Buffer (Qiagen) added with 1% of β-mercaptoethanol that permits to separate cellular components. The lysate was then placed into RNasy MiniElute spin supplied by the manufacturer. Any possible genomic DNA contamination was eliminated by DNase I incubation. RNA was completely purified by several steps of centrifugation in appropriate RNasy MiniElute spin column, where the column was saved and the flow-through discarded. In the final step the RNasy MiniElute columns was transferred to the Eppendorf RNase-free tube and centrifuged once again by adding the appropriate volume of RNase-free water directly to the center of the spin column to elute the RNA. Taq-Man Reverse Transcription was then

performed to generate cDNA. Following the manufacturer's instruction, we used a master mix containing: M-MLV retro-transcriptase enzyme (Moloney Murine Leukemia Virus), MgCl2 to activate the enzyme, random hexamers were used as primers for reverse transcriptase, dNTPs deoxynucleotides triphosphates for the elongation of cDNA strength and RNAse inhibitors. The reaction was carried out with the following setup: 25°C 10', 48°C 30', 95°C 3' in a final volume of 50µl. Primers and probes used for real-time RT-PCR were purchased from Life Technologies, the reaction was performed using ABI PRISM® 7900HT with FAST protocol.

Co-cultures and proliferation assay

With regard to experiments aimed at evaluating lymphocyte proliferation, 24h before the experiment, mesenchymal cells were detached from flask and plated at different ratio (1:5, 1:10, 1:50 and 1:100, compared to 100,000 T cells that will be plated) in a 96 flat-bottomed well plate. After 2 hours in adherence, TNF-α and IFN-γ were added. After this over-night stimulus, DMEM was discarded and T cells, different inhibitory stimuli (catalase, 1-methyl tryptophan and anti-PD-L1) and anti-CD3/CD28 plus IL2 were added to the wells.

On day 3, cultures were pulsed for 8 h with 0.5 μCi (0.0185 MBq) of ^{3}H-TdR (Amersham, UK) harvested, and radionuclide uptake was measured by scintillation counting.

Adhesion assay

To evaluate CD4+ T cells adhesion, we pre-treated MSCs as mentioned above, plating cells in a 6 well plate. The next day, CD4+ T cells were added. After few hours we removed non-adherent T cells by gentle shaking the plate (to avoid MSCs detachment) and discarding the culture medium. Then, we recovered all the remaining cells washing the well with PBS, the absolute number of CD4+ T cells was determined by MACSQuant Analyzer (Miltenyi Biotech).

Transswell assay

To assess if the inhibitory action of MSCs, was contact dependent or not, we performed a co-culture experiment on a Transwell® plate (Corning) with pores of the size of 0,4μm, thus not allowing T cells migration. Briefly, MSCs were pre-treated as mentioned before and plated on the bottom of the Transwell plate. On the day after, we added CD4+ T cells or over the membrane or directly in contact with MSCs. After 5 days, T cells were recovered and analysed.

Statistics

Statistical analysis was performed using the Student T-test. The differences were considered statistically significant for $p \leq 0.05$.

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