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# **Contribution of tumor-associated neutrophils in the course of cutaneous squamous cell carcinoma : neutrophils and cancer**

Sokchea Khou

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# THÈSE DE DOCTORAT

UNIVERSITÉ  
CÔTE D'AZUR 

ÉCOLE DOCTORALE  
SCIENCES DE LA VIE  
ET DE LA SANTÉ



## Contribution des neutrophiles infiltrant les tumeurs dans la progression des carcinomes épidermoïdes cutanés

Neutrophiles et cancer

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**Présentée en vue de l'obtention  
du grade de docteur en discipline  
d'Université Côte d'Azur**  
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# Contribution des neutrophiles infiltrant les tumeurs dans la progression des carcinomes épidermoïdes cutanés

Neutrophiles et cancer

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## Contribution des neutrophiles infiltrant les tumeurs dans la progression des carcinomes épidermoïdes cutanés

Les carcinomes cutanés constituent les cancers les plus fréquents chez l'homme et leur incidence est en constante croissance. Il en existe deux principaux types: les carcinomes baso-cellulaires et les carcinomes épidermoïdes. Les facteurs de risque sont principalement l'exposition aux rayons UV et l'immunosuppression. Ils sont traités par chirurgie ou par radiothérapie mais peuvent parfois évoluer vers des formes incurables. De nouvelles alternatives thérapeutiques sont donc nécessaires.

L'immunothérapie est une récente révolution dans le traitement des cancers. Ceci vise à réactiver l'immunité des patients cancéreux. Les carcinomes cutanés pourraient en bénéficier car ils se développent lors de situations d'immunosuppression.

L'immunosurveillance implique à la fois les cellules immunitaires et le microenvironnement tumoral comme le stroma. Lorsque les réponses anti-tumorales, notamment médiées par les lymphocytes T CD8<sup>+</sup> sont efficaces, on parle de phase d'élimination. Puis vient une phase d'équilibre où la tumeur reste stable et enfin, lors de la phase finale d'échappement, des mécanismes d'immunosuppression permettent à la tumeur de croître.

Les neutrophiles, des cellules immunitaires de type myéloïde, sont recrutées très rapidement aux sites d'inflammation et au sein des cancers. Une méta-analyse récente sur 39 types de cancers humains a pu associer ces cellules aux plus mauvais pronostiques cliniques. Elles sont impliquées dans des fonctions anti-tumorales et pro-tumorales. Cette polarisation semble induite respectivement par l'interféron de type I et le TGF- $\beta$ . Leur rôle lors de cancers et en particulier dans les carcinomes cutanés reste encore largement incompris. Notre objectif a été de caractériser les fonctions des neutrophiles et leur contribution au développement des carcinomes épidermoïdes. Pour cela, nous avons utilisé premièrement, un modèle de carcinogénèse cutanée chimio-induite. La tumorigénèse est séquentielle et donc très représentative du carcinome cutané chez l'homme. Dans ce modèle, nous observons une infiltration massive des neutrophiles au stade précancéreux et cancéreux. Une analyse de l'expression génique des neutrophiles isolés des lésions précancéreuses et cancéreuses et des peaux environnant ces lésions a été réalisée, qui montre une signature génique spécifique des neutrophiles des lésions, comparée aux peaux environnantes. Des comparaisons d'expression génique différentielle illustrent que les neutrophiles des lésions possèdent des fonctions pro-tumorales comparés aux neutrophiles des peaux.

Deuxièmement, nous avons mis en place un modèle de greffe intradermique d'une lignée de carcinome épidermoïde. Une déplétion spécifique des neutrophiles retarde significativement la croissance tumorale, ce qui confirme le caractère pro-tumoral des neutrophiles. Nous avons caractérisé les mécanismes mis en jeu, qui incluent la génération de ROS et iNOS favorisant la croissance tumorale et une suppression de l'activation et de la prolifération des lymphocytes T CD8<sup>+</sup> anti-tumoraux. Les neutrophiles produisent de l'arginase 1 qui dégrade l'arginine et inhibe la prolifération

des lymphocytes T. Par ailleurs, l'environnement tumoral induit l'expression de PD-L1 en surface des neutrophiles et de PD1 en surface des lymphocytes T CD8+ et CD4+. Ceci suggère que l'interaction PD-L1/PD1 contribue à l'immunosuppression. De plus, une corrélation positive et significative a été observée entre la taille des tumeurs et la fréquence des neutrophiles exprimant PD-L1 au sein de ces tumeurs.

Au vu de ces résultats, il semble intéressant d'évaluer les immunothérapies bloquant l'interaction PD-L1/PD1 dans le traitement des carcinomes cutanés. Ces traitements pourraient être combinés avec ceux qui bloquent le recrutement ou les fonctions des neutrophiles. Il reste à évaluer si la fréquence de neutrophiles exprimant PD-L1 peut être un bon marqueur de prédiction de la réponse aux immunothérapies anti-PD-L1 ou anti-PD1.

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**Mots clés :** Carcinomes cutanées, Neutrophiles, Protumoral, Immunosuppression, PD-L1, PD-1, Transcriptome

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### **Contribution of tumor-associated neutrophils in the course of Cutaneous Squamous Cell Carcinoma**

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Non-melanoma skin carcinomas are the most frequent cancers in Human and their incidence is constantly increasing. Two main types exist: the cutaneous basal cell carcinoma (cBCC) and the cutaneous squamous cell carcinomas (cSCC). Risk factors include sun radiation and immunosuppression. These cancers are mainly treated with surgery and radiotherapy but they can reach an incurable stage. For this reason, novel therapeutic alternatives are needed.

At present, immunotherapies constitute a revolution in the treatment of cancers. Its mechanism of action relies on the stimulation of the immune system of cancer patients, so that they develop efficient anti-tumoral immune responses. cSCC may benefit from this type of treatment as they generally develop in the context of an immunosuppression.

Immune surveillance involves both immune cells and the tumor microenvironment, in particular the stroma. During the elimination phase, the anti-tumoral responses, mediated mainly by CD8+ T lymphocytes, are efficient. Then, there is an equilibrium phase in which the tumor is stable before the escape phase, when the tumor can evade immune surveillance and grow.

Our research interest focused on neutrophils, a subset of myeloid cells that are very rapidly recruited to the sites of inflammation and inside tumors. A recent meta-analysis of 39 human malignancies showed that neutrophils are associated with the worst clinical outcome. Neutrophils harbor both anti- and pro-tumoral functions. This polarization seems to be dependent on type I interferon and TGF- $\beta$ , respectively. It remains to establish the exact role played by neutrophils in cancer and specifically in skin carcinomas. The aim of our research was to further characterize the functions and the contribution of neutrophils to the development of cutaneous squamous cell carcinomas. We first used a chemically-induced skin carcinoma mouse model that recapitulates the different stages of skin carcinoma development in Human. In this model, we saw a massive infiltration of neutrophils at the precancerous and cancerous stages. We

performed transcriptomic analysis of highly purified neutrophil populations from precancerous, cancerous lesions and from the surrounding skin controls. These data revealed a specific gene signature in neutrophils from lesions compared to surrounding skins. Differential gene expression analysis identified a pro-tumoral phenotype for neutrophils infiltrating lesions compared to skins.

In a second approach, we studied the growth of a cSCC cell line grafted in the dermis of mice. Specific depletion of neutrophils significantly delayed tumor growth, thus indicating that neutrophils were pro-tumoral. Mechanisms of action included the production of ROS and NO that favor tumor growth and the immune suppression of anti-tumoral responses mediated by tumor-associated CD8+ T cells. In the tumor, neutrophils produced arginase 1 which catalyzes the degradation of arginine, thus inhibiting the proliferation of CD8+ T cells.

In addition, we found that the tumor microenvironment induced PD-L1 expression at the cell surface of neutrophils and concomitantly, PD1 on CD8+ and CD4+ T cells. These results suggested that PD-L1/PD1 interaction triggers immune suppression and contributes to SCC progression. Indeed, a positive and significant correlation was observed between tumor size and frequencies of PD-L1-expressing neutrophils inside tumors.

Collectively, these results suggest that it is relevant to assess immunotherapies that block PD-L1/PD1 interaction for the treatment for cSCC. These approaches could be combined with treatments that aim to block the recruitment or inhibit neutrophils. Moreover, it remains to evaluate whether the frequency of PD-L1-expressing neutrophils could constitute a good predictive marker of the response to anti-PD-L1 and anti-PD1 immunotherapies.

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**Keywords** : Cutaneous squamous cell carcinomas, Neutrophils, Protumoral, Immunosuppression, PD-L1, PD-1, Transcriptomic analysis

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## List of abbreviation

AK (Actinic Keratosis)  
APC (Antigen Presenting Cell)  
ADCC (Antibody-Dependent Cellular Cytotoxicity)  
ATP (Adenosine Tri-Phosphate)  
ADP (Adenosine Di-Phosphate)  
AMP (Anti-Microbial Peptide)  
BCC (Basal Cell Carcinoma)  
C3aR (Complement C3a Receptor)  
CC (Colon Cancer)  
cBCC (Cutaneous Basal Cell Carcinoma)  
cSCC (Cutaneous Squamous Cell Carcinoma)  
cDC (conventional DC)  
DC (Dendritic Cell)  
DAMP (Damage-Associated Molecular Pattern)  
ECM (ExtraCellular Matrix)  
EGF (Epidermal Growth Factor)  
fMLP (N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine)  
G6P (Glucose 6-Phosphate)  
G-CSF (Granulocyte Colony-Stimulating Factor)  
GPCR (G Protein-Coupled Receptor)  
HNC (Head and Neck cancer)  
HPV (Human PapillomaVirus)  
IEC (IntraEpidermal Carcinoma)  
IMC (Immature Myeloid Cell)  
iNOS (inducible Nitric Oxide Synthase)  
LPS (LipoPolySaccharide)

LC (Langerhans Cell)  
Lox-1 (Lectin-type oxidized LDL receptor-1)  
MM (Malignant Melanoma)  
MMP (Matrix MetalloProtease)  
MDSC (Myeloid-Derived Suppressor Cell)  
MRSA (Methicillin-Resistant Staphylococcus Aureus)  
NMSC (Non-Melanocytic Skin Cancer)  
NK (Natural Killer)  
NCR (Natural Cytotoxicity Receptor)  
NO (Nitric Oxide)  
NSCLC (Non-Small Cell Lung Cancer)  
NET (Neutrophil Extracellular Trap)  
nTreg (natural Treg)  
Ocn (Osteocalcin-Expressing)  
SCC (Squamous Cell Carcinoma)  
ROS (Reactive Oxygen Specie)  
Treg (regulatory T cell)  
TAM (Tumor-Associated Macrophage)  
TAN (Tumor-Associated Neutrophil)  
TGF- $\beta$  (Transforming Growth Factor  $\beta$ )  
TLR (Toll-Like Receptor)  
TCR (T Cell Receptors)  
PPP (Pentose Phosphate Pathway)  
PAMP (Pathogen-Associated Molecular Pattern)  
PDGF (Platelet-Derived Growth Factor)  
RAS (Renin-Angiotensin System)  
PHA (PhytoHaeMagglutinin)  
PD-L1 (Programmed Death-Ligand 1)  
UV (UltraViolet)

VEGF (Vascular Endothelial Growth Factor)

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# **INTRODUCTION**

# 1. Cancer

According to World Health Organization, Cancer is the second leading cause of death worldwide. Approximately 70% of deaths from cancer occur in low- and middle-income countries, where the diagnostic and treatment are insufficiently taken in charge by public health service. In 2018, the estimation of cancer-related death is up to 9.6 million. Globally, about 1 in 6 deaths is due to cancer. The most common cancers are: Lung (2.09 million cases), Breast (2.09 million cases), Colorectal (1.80 million cases), Prostate (1.28 million cases), Skin (1.04 million cases), Stomach (1.03 million cases). (<https://www-who-int.gate2.inist.fr/news-room/fact-sheets/detail/cancer>)

## 1.1. Causes

Cancer is generally a multifactorial polygenic disease. Some risk factors have been identified and include:

- Genetic/hereditary causes: Cancer is caused by modifications of genes that control cell growth and division (detailed in the later chapter in this manuscript). These genetic changes can be inherited from parents if they are present in the germ cells, known as hereditary cancer syndromes, which account for 5 to 10% of all cancers. Several mutations in specific genes have been reported such as inherited mutations in the *BRCA1* and *BRCA2* genes, which are linked to hereditary breast and ovarian cancer and Lynch syndrome.
- Physical carcinogens, such as ultraviolet and ionizing radiation
- Chemical carcinogens, such as tobacco and alcohol
- Infections from certain viruses, bacteria, or parasites (for example Epstein-Barr Virus, Hepatitis B Virus and Hepatitis C Virus, Human Papillomaviruses, *Helicobacter pylori*, *Schistosoma hematobium*,....) can cause or promote cancer by directly affecting cell growth, or by the generation of a chronic inflammation.
- Hormone, in particular estrogen, is known to promote tumor development in some human cancer. In fact, excessive exposure to estrogen alone or estrogen combined with

progesterone is likely associated to high risk of developing endometrial cancer and breast cancer respectively.

- Immunosuppression due to medication or infection by HIV for example weakens the immune system and augments the risk of cancer development
- Obesity is linked to an increase of cancer development, in contrast, a healthy diet and being physically active may reduce risk of some cancer and this is linked in part with chronic inflammation.
- Aging is an important factor in forming cancer in general. This is supported by the fact that the median age of cancer diagnosis is 66 years old according to the most recent studies.
- Chronic inflammation, resulting not only from infection or obesity but also from abnormal immune responses is an important risk factor (for example, inflammatory bowel diseases are linked with colon cancer)

(<https://www.cancer.gov/about-cancer/causes-prevention/risk>;

<https://www.cancer.gov/about-cancer/causes-prevention/genetics>)

## **1.2. Types of cancer**

Cancer can develop in any type of cells and location in the body, generating different types of cancer, which can be distinguished by their behavior and response to treatment. According to the type of cells from which tumors arise, there are three main types of cancer:

- Carcinomas are solid tumors of epithelial cells, representing 80-90% of cancers.
- Sarcomas are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue.
- Hematological malignancies including leukemia and lymphoma arise from blood-forming cells and cells of the immune system.

Solid tumors are also classified into benign or malignant tumors. Benign tumor is the one that remains confined to its original location, neither invading surrounding normal tissue nor spreading to distant body sites; and malignant tumor is the one that is capable of both invading

surrounding normal tissue and spreading throughout the body via the circulation system, called “invasive cancer and metastasis”.

### **1.3. Cancer development**

Cancer development is a multistage process in which a single transformed cell expands into a clone of malignant cells, forming subsequently a tumor. The phenomena can happen over many years and involves several genetic changes, which occurs during the progeny of the transformed cells. The accumulation of those modifications over time will then establish a malignant phenotype, resulting in abnormal cellular growth capacity (Zhang et al., 1997).

Under normal condition, cell division and growth is positively regulated by a group of proteins coded by genes, called proto-oncogenes; and is negatively controlled by tumor suppressor genes. The maintenance of genome stability is vital and guaranteed by multiple mechanisms and checkpoints that resolve DNA damage. During cancer development, genetic instability leads to two cellular processes that result in a deregulation of cell apoptosis, survival and proliferation. One is a transformation of normal proto-oncogenes to oncogenes and two is a loss of tumor suppressor gene function (Amy Y. Chow et al., 2010). As a result, a relatively restricted number of key pathways have been shown to be altered and are critically involved in tumor formation and progression.

Proto-oncogenes are transformed into oncogenes by three genetic mechanisms. The first genetic alteration of this transformation is mutation. This later will permanently activate proteins that normally interchange between active or inactive states, leading to an uncontrollable excess of their functions. For example, point mutations are frequently detected in the ras family of proto-oncogenes (*K-ras*, *H-ras*, and *N-ras*), and mutation in *K-ras* in particular predominates in carcinomas. A second type of genetic modification is a chromosomal rearrangement. These rearrangements consist mainly of chromosomal translocations and, less frequently, chromosomal inversions, creating fusion genes when the chromosomal breakpoints fall within the loci of two different genes. Fusion genes present an altered regulation of protein

expression (ie *BCR/ABL*). A third possibility to generate oncogenes is amplification of expression from multiple copies of proto-oncogenes that already exist in the cells. A common gene amplified in tumors is the transcription factor *c-MYC*. (Pierotti et al., 2003).

Tumor suppressor genes code for negative regulator proteins that normally operate to restrict cellular growth and division, promote programmed cell death (apoptosis) and repair damaged DNA. Best-known tumor suppressor genes are *RB1* (retinoblastoma), *Tp53* (*Tumor protein 53*), and *Tp21*. Mutation of one of negative regulators might make cells incapable to halt the cell cycle if there is a problem. In fact, mutated *Tp53* genes have been found in more than a half of human tumor cells. Without fully functional protein 53 (*p53*) encoded by *Tp53* gene, the G1 checkpoint of interphase is severely compromised and the cell proceeds directly from G1 to S regardless of internal and external conditions. At the completion of this shortened cell cycle, mother cell generates two daughter cells that have inherited the mutated *p53* gene. The loss of *p53* function can also result in inadequate production of *p21* (Cooper et al., 2000).

## **1.4. Skin cancer**

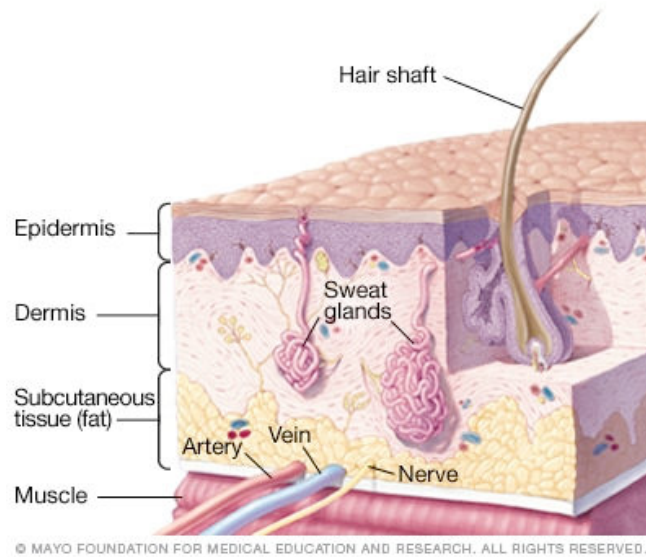
Skin cancers are the most common malignancies worldwide. One in every three cancers diagnosed is a skin cancer according to World Health Organization. The main factor for developing skin cancer is exposure to ultraviolet (UV) radiation.

### **1.4.1. Skin structure**

Skin is the largest organ of the body that provides its outer protection. It accounts for about 15% of the total body weight. Skin has many vital functions. It serves as a protection against external physical, chemical and biological injuries and also ensures the detection of heat, cold, pain and touch sensations. It participates in the process of thermoregulation (Kolarsick et al., 2011).

To fulfill these functions, the skin is composed of three layers: the epidermis, the dermis and the hypodermis as show in Figure 1 (Kanitakis et al., 2002; Urmacher et al., 1990).

**Figure 1** Cross-section of skin showing the structure of three layers of skin: epidermis, dermis and hypodermis (subcutaneous) from MayoClinic.com



#### 1.4.1.1. Epidermis

**The epidermis** is a stratified, squamous epithelium layer. This layer comprises about 90% of keratinocytes and 10% of melanocytes, Merkel cells, Langerhans Cells (LCs, epidermis dendritic cells), and some T cells.

##### ➤ Keratinocytes

Keratinocytes produce keratins, fibrous proteins that make filaments protecting epithelial cells from mechanical stress but also giving the skin its hardness and waterproof properties. In addition, even though, they are not immune cells, keratinocytes play an important role in immune defense. They are the main source of antimicrobial peptides (AMP). These small molecules are the first line of defense against microorganisms. Keratinocytes also express some Toll-like receptors (TLR) whose activation leads to a production of pro-inflammatory cytokines that subsequently stimulate Langerhans cells (LC) located nearby. According to keratinocyte structures, epidermis is divided into 4 layers: basal layer (stratum germinativum); squamous layer (stratum spinosum); granular layer (stratum granulosum), and cornified layer (stratum

corneum). These different structures develop during their differentiation, a process that occurs as the cells develop from the basal layer and then migrate upward to the surface of the skin up to the cornified layer. This process is called keratinization in which the keratinocyte first passes through a synthetic and then a degradative phase. The dynamic of keratinocyte differentiation makes epidermis a continually renewing layer.

➤ **Melanocytes**

Melanocytes are pigment-synthesizing cells. They are responsible for the production and secretion of melanin pigment that give a color and protection from UVs to skin.

➤ **Merkel cells**

These cells are part of the tactile-end organs in the skin with type 1 mechano-receptors.

➤ **Langerhans cells**

LCs originate from the bone marrow and migrate to the epidermis where they are the first immune cells that come into contact with microorganisms. Their primary role is to present antigen. They are characterized by expression of the receptor Langerin (CD207) (Valladeau et al., 2000). In human, LCs express the lipid-presenting molecule CD1a. In mice, LCs can be characterized by expression of CD11b, CD207, Epcam (CD326) and CD24 (Guilliams et al., 2014, 2016).

➤ **T cells**

Some T cells reside in the epidermis, close to LCs. They include CD8<sup>+</sup> αβ T cells, but also Dendritic epidermal T cells (DETCs) expressing γδ TCR and NKT cells (Matejuk et al., 2018; Pasparakis et al., 2014). DETCs have been described in mice but not in human.

#### **1.4.1.2. Dermis**

The dermis lies beneath the epidermis layer. It is thicker than the epidermis and is composed of a dense network of connective tissue (collagen and elastin). This layer is organized into fibers of

different sizes and properties. This network generates a sort of extracellular matrix that varies in a depth-dependent manner and undergoes turnover and remodeling. The extracellular matrix contains blood and lymphatic vessels, nerves, the bottom part of the hair follicles and sweat glands (Kolarsick et al., 2011).

Different cell types are found in this layer (Pasparakis et al., 2014)

➤ **Fibroblasts**

Fibroblasts are the main cell population that resides in the dermis. Their principal function is to synthesize or degrade the extracellular matrix. They are also a key actor in dermo-epidermal interactions.

➤ **Macrophages**

They become terminally differentiated cells in dermis. As antigen presenting cells, macrophages phagocytose cellular debris and pathogens then process them and present antigens to lymphocytes and other immune cells to trigger a specific immune response.

➤ **Dermal dendritic cells**

They are present in the deeper dermis. Several subpopulations have been identified, cDC1 and cDC2 in mice respectively characterized by the expression of CD103 and XCR1 for cDC1 and CD11b and Sirp- $\alpha$  for cDC2 (Guilliams et al., 2014, 2016). In human, they are identified by CD11c and high levels of HLA-DR and characterized by the absence of CD14 (Haniffa et al., 2012). They constitute a second line of defense that triggers immune response against cutaneous or circulating antigens behind LCs.

➤ **Plasmacytoid dendritic cells**

They are another type of dendritic cells that reside in the skin. Sharing similarities to B cells, these antigen-presenting cells express a high level of MHCII. In human, they express the marker CD123, CD303, CD304 while in mice they are identified by the expression of Siglec H. They are known to be able to secrete a large amount of type I interferon during viral infection.

➤ **Mast cells**

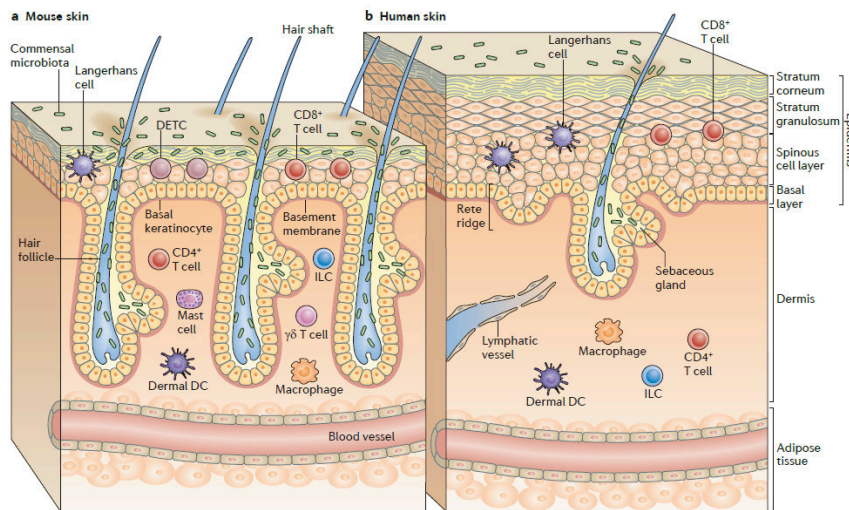
Located mostly around blood vessels, these secretory cells are specialized in releasing their intracellular granules rich in histamine and heparin in response to physical (light, cold, heat, acute trauma), chemical and immunological stimuli. This degranulation induces vasodilatation, dermal edema, followed by recruitment into the dermis of inflammatory cells such as neutrophils, eosinophils and basophils.

➤ **T cells**

Skin is the largest reservoir of T cells in the whole body. More than 95% of them have a memory phenotype, suggesting that they have already experienced their antigens. Several types of T cells have been identified including CD8<sup>+</sup> and CD4<sup>+</sup> αβ conventional T cells, Th17 cells, γδ T cells, DETC (in mice).

The composition and localization of immune cells in mice and human skin is illustrated in Figure 2.

**Figure 2.** Composition and localization of immune cells in mouse and human skin from (Pasparakis et al., 2014)



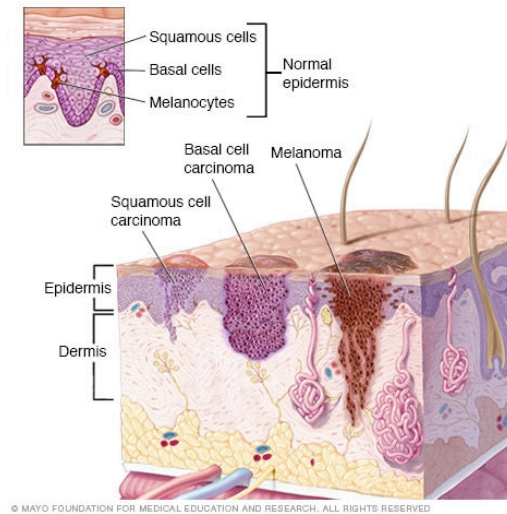
### 1.4.1.3. Hypodermis

It is the deepest layer of the skin, which contains a collagen fiber network and adipocytes. It acts as a main storage site for fat and therefore energy. There are many blood and lymphatic vessels and nerves passing through this area (Kolarsick et al., 2011).

### 1.4.2. Different types of skin cancer

There are various types of skin cancer. They are named according to the cells from which they arise and their clinical behavior. They can be divided into two main groups: non-melanoma skin cancer (NMSC) arising from transformed keratinocytes, and malignant melanoma (MM) arising from transformed melanocytes (Centre et al., 2013). The location and morphology of keratinocytes and melanocytes with their related carcinogenesis are illustrated in Figure 3.

**Figure 3.** The location and morphology of the keratinocytes and melanocytes of the normal and cancerous epidermis, from MayoClinic.com



#### 1.4.2.1. Malignant melanoma

MM account for only 4% of skin cancers, though it has the highest mortality rate among skin cancers. It is a malignancy of the melanocytes, which are pigment-producing cells. The principal

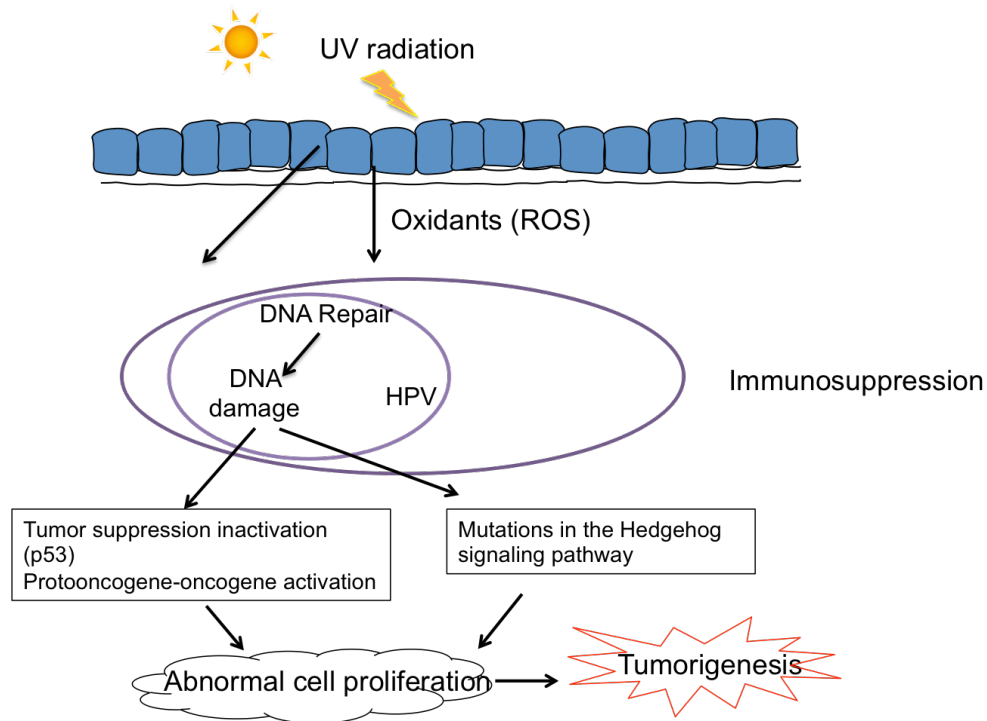
risk for developing melanoma is excessive exposure to UV radiation. As white fair skin is known to be more sensitive than dark skin, a highest incidence of MM is found in the Caucasian population.

In about 50% of melanoma, the mutated proto-oncogene is found to be *BRAF* gene, encoding for a group of proteins called BRAF kinase. BRAF kinase plays a role in regulating the mitogen-activated protein kinase/extracellular signal-regulated kinase(MAP/ERK) signaling pathway, which affects cell division and differentiation. This mutation leads to uncontrolled proliferation of melanocytes. In some patients with familial association, mutations mostly affect *Tp53* gene, the tumor suppressor gene described previously. Mutation has also been found to occur in the Cyclin-dependent kinase inhibitor 2A (*CDNK2A*) gene (Centre et al., 2013). The rest of melanoma involve mutations of *NRAS* (15-20%), *PTEN*, *MYC*, *MITF*, *ERBB4* and *NEDD9* (Flaherty et al., 2012; Wilson and Nathanson et al., 2012).

#### **1.4.2.2. Non-melanoma skin cancer**

NMSC is the most common form of cancers. The incidence continues to increase since 1960 worldwide. Two main types of NMSC are Cutaneous Basal Cell Carcinoma (cBCC) and Cutaneous Squamous Cell Carcinoma (cSCC). NMSC are caused by both environmental factors such as UV radiation, chemical exposure and host factors including HPV (Human Papillomavirus) infection, genetic susceptibility, and immunosuppression (summarized in Figure 4).

**Figure 4.** Carcinogenic pathways associated with cutaneous basal cell carcinoma and cutaneous squamous cell carcinoma



#### ➤ Cutaneous Basal Cell Carcinoma

cBCC is a cancer of the basal cells of the epidermis. It is the most frequent skin cancer, making up about 80% of NMSC. cBCC develop *de novo*, are slow growing tumors and very rarely metastasize. About 90% of cBCC genesis involves mutations in the Hedgehog signaling pathway, a tumor suppressor. This intracellular pathway is a key regulator of cell growth and differentiation during embryogenesis (Ingham and McMahon et al., 2001). The extracellular hedgehog protein binds with patched homolog 1 (PTCH1), a transmembrane receptor. This interaction prevents downstream PTCH1-mediated inhibition induced by smoothed homolog (SMO) signaling. In normal adult tissues, both PTCH-1 and SMO are inactivated. Mutations in either PTCH-1 or SMO result in constitutive activation of the Hedgehog signaling pathway, thus allowing unrestricted proliferation of epidermal basal cells. Mutations in the *Tp53* gene are present in more than 50% of cases, but is more related to the progression than the initiation of cBCC (Xie et al., 1998).

Clinically, cBCC is divided into four subtypes: Superficial, Nodular ulcerative, Pigmented and Morpheaform (Dubas and Ingraffea et al., 2013). The nodular ulcerative cBCC represents between 50% and 85% of all biopsied cBCC lesions (Chinem and Miot et al., 2011). Histology for most of those clinical subtypes shows the basaloid, basophilic staining cells budding downward from the epidermis with palisading of peripheral cells.

The subtyping is very important for clinical management. The treatment of choice for the lesions involving head and neck is Mohs micrographic surgery (MMS) because the technique can preserve healthy tissues in areas at high risk for recurrence. When tissues preservation is unessential, a nonaggressive BCC can be removed by a local excision with 4mm to 5mm margins. There are also nonsurgical treatment options such as radiation, imiquimod (agonist of Toll-like receptor 7), photodynamic therapy (PDT), ingenol mebutate (PEP005, a natural substance from plant *Euphorbia peplus*), and vismodegib (a systemic inhibitor of Hedgehog signaling) (Dubas and Ingraffea et al., 2013).

#### ➤ **Cutaneous Squamous Cell Carcinoma**

cSCC is a malignant neoplasm of keratinocytes of the epidermis and account for 20% of non-melanoma skin cancer. cSCC arise from precursor lesions of actinic keratosis (AK) and Bowen's disease, and develop slowly from a multistep accumulation of genetic damage. 5–10% of them can metastasize to the lymph nodes at an early stage, making cSCC more aggressive and responsible for many deaths.

cSCC carries more mutations than other cancers, mainly due to the exposure of the skin to UV light. Inactivation of the key tumor suppressor gene *Tp53*, occurs in nearly 90% of cSCC lesions (Brash et al., 1991). Others mutations occur in genes like *CDKN2A*, *RAS*, *NOTCH1*, *KMT2C* (Pickering et al., 2014).

Immunosuppression and human papillomavirus (HPV) infection are other risk factors for the development of cSSC. HPVs are a group of small, non-enveloped, double-stranded DNA viruses. HPVs infect cutaneous and mucosal epithelia (McLaughlin-Drubin et al., 2012). 99% of cervical cancers are positive for HPV 16 DNA suggested that HPVs are the causative agent (Walboomers et al., 1999). The mechanism might involve viral proteins E6 and E7 that act as oncoproteins by

inhibiting tumor suppressor protein *p53* and retinoblastoma (Rb) family, respectively (Dyson et al., 1989; Münger et al., 1989; Scheffner et al., 1990; Werness et al., 1990). A meta-analysis of data from the literature reported a clear association between HPV (5 and 8) and cSCC with a high prevalence in immunosuppressed patients compared to immunocompetent patients (Wang et al., 2014).

cSCC lesions show full-thickness epidermal dysplasia. cSCC may arise *de novo* or from AK, defined as intraepithelial proliferation of atypical keratinocytes, generating premalignant precursor lesions that show partial-thickness epidermal dysplasia. Histologically, cSCC is categorized by the thickness of epidermal invasion and degree of keratinocyte differentiation, into three categories: well differentiated, moderately differentiated, and poorly differentiated. This degree of differentiation inversely correlates with tumor aggressiveness (Walboomers et al., 1999).

Similar to cBCC, many options, both surgical and nonsurgical, exist for the treatment of cSCC. The choice is based on the patient's age and functional status, surgical candidacy, lesion size, location, depth of invasion, level of differentiation, perineural or perivascular invasion, recurrence of previous cSCC, and immunosuppression (Rowe et al., 1992).

## **2. The tumor microenvironment**

In solid tumors, cancer cells grow in a microsystem called the tumor microenvironment (TME). It has long been thought that genetic and epigenetic changes were initiating and driving the carcinogenic process, giving the cells a growth advantage. Changes in the TME were viewed as playing a secondary role. This view is now challenged. D. Hanahan and R. A. Weinberg have defined "The hallmarks of Cancer", listing the functional capabilities that allow cancer cells to survive, proliferate and disseminate (Hanahan and Weinberg et al., 2000, 2011).

## **2.1. Tumor microenvironment composition**

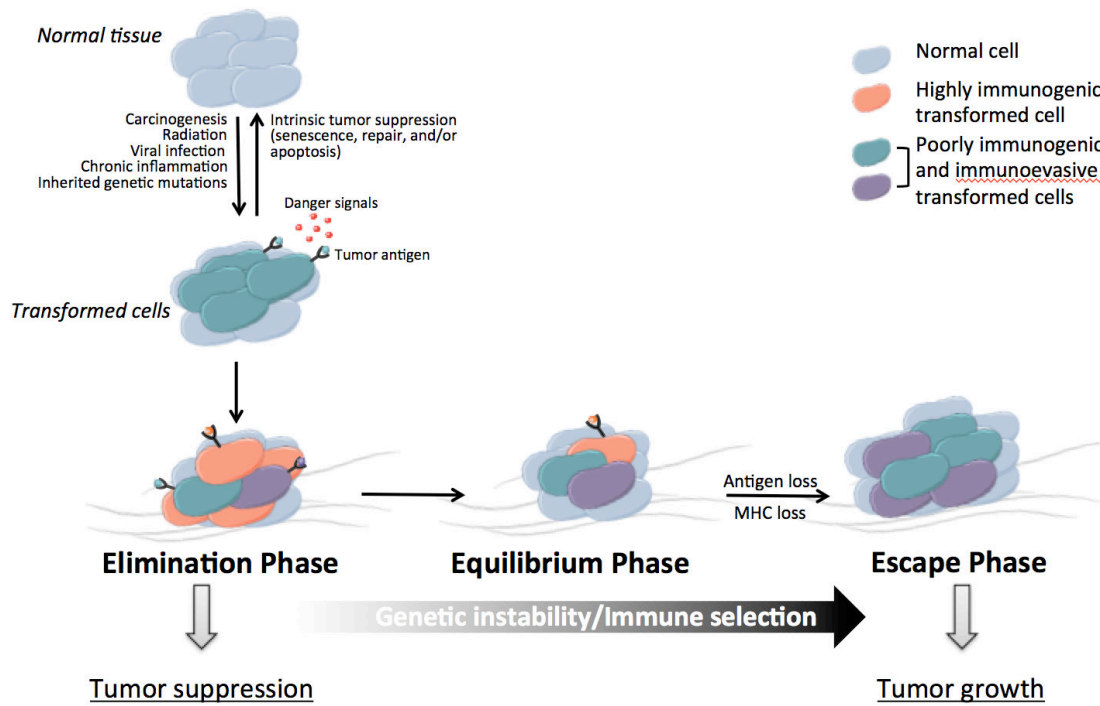
TME refers to the parenchyma where lie tumor cells and the stroma which contains several types of non-malignant cells including blood and lymphatic endothelial cells (BECs and LECs), mesenchymal stem cells (MSC), cancer-associated fibroblasts (CAFs), pericytes and several types of immune cells. TME is also composed of the extracellular matrix (ECM) and soluble mediators produced by the above cellular components. The composition of TME varies from one type of tumor to another. Frequently, tumors are abnormally vascularized with dysfunctional vessels. Blood and lymphatic endothelial cells present in TME are involved in forming this new vascularization system favoring a neoplastic progression of tumor cells and infiltration of immune cells (Turley et al., 2015). This favor the establishment of a chronic inflammation promoting tumor growth (Mantovani et al., 2008). MSC are a heterogeneous population of multipotent precursor cells that are recruited into tumors where they are further differentiated into pericytes and CAF (Mishra et al., 2008). CAF represent the majority of non-hematopoietic cells in TME. They are characterized by an activated, highly contractile, myofibroblastic phenotype and normally identified by expression of vimentin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and fibroblast activation protein (FAP). Their abundance in TME is often associated with a poor prognostic (Ha et al., 2014). Indeed, CAF contribute to initiate and favor tumor growth through remodeling of ECM and stimulation of tumor cell proliferation via the secretion of growth factors. Overall, it is now recognized that the stroma is a key player that regulates tumor growth and metastasis, thus contributing to malignancy.

## **2.2. Cancer immunosurveillance**

The concept of immunosurveillance has been debated since the 1950s by Sir Macfarlane Burnet and Lewis Thomas (Burnet et al., 1957) but was really acknowledged in the 1990s-2000s (Dunn et al., 2002; Hanahan and Weinberg et al., 2000). It was found that the accumulation of genetic alterations results in the generation of neoantigens that differentiate tumor cells from their normal counterparts. These neoantigens are presented as peptides bound to Major

Histocompatibility Complex (MHC) class I molecules to CD8<sup>+</sup> T lymphocytes, thus triggering a cytotoxic immune response (Boon et al., 1994). The discovery of natural killer (NK) cells and the use of knock-out mice such as IFN- $\gamma$ <sup>-/-</sup>, perforin<sup>-/-</sup> or RAG<sup>-/-</sup> mice also contributed to further identified anti-tumor responses (Dunn et al., 2002). The immune response is dynamic and has a central role in shaping tumor progression. At the beginning of cancer development, neoplastic cells induce an immune response leading to the elimination of developing tumors. However, this elimination is eventually incomplete resulting in the editing of tumors by the immune cells to escape from immunity. To describe these various stages, Schreiber et al proposed the concept of “immunoediting or immunosurveillance” (Dunn et al., 2002). Immunoediting is described as a process of malignant progression based on interaction between tumor cells and immune cells, which is divided into three phases: (1) ELIMINATION, where transformed cells are eliminated by the immune system; (2) EQUILIBRIUM, where transformed cells are held under control but are not eliminated by the immune system; and (3) ESCAPE, where transformed cells overcome the immune response and form a tumor (Dunn et al., 2002) as illustrated in Figure 5.

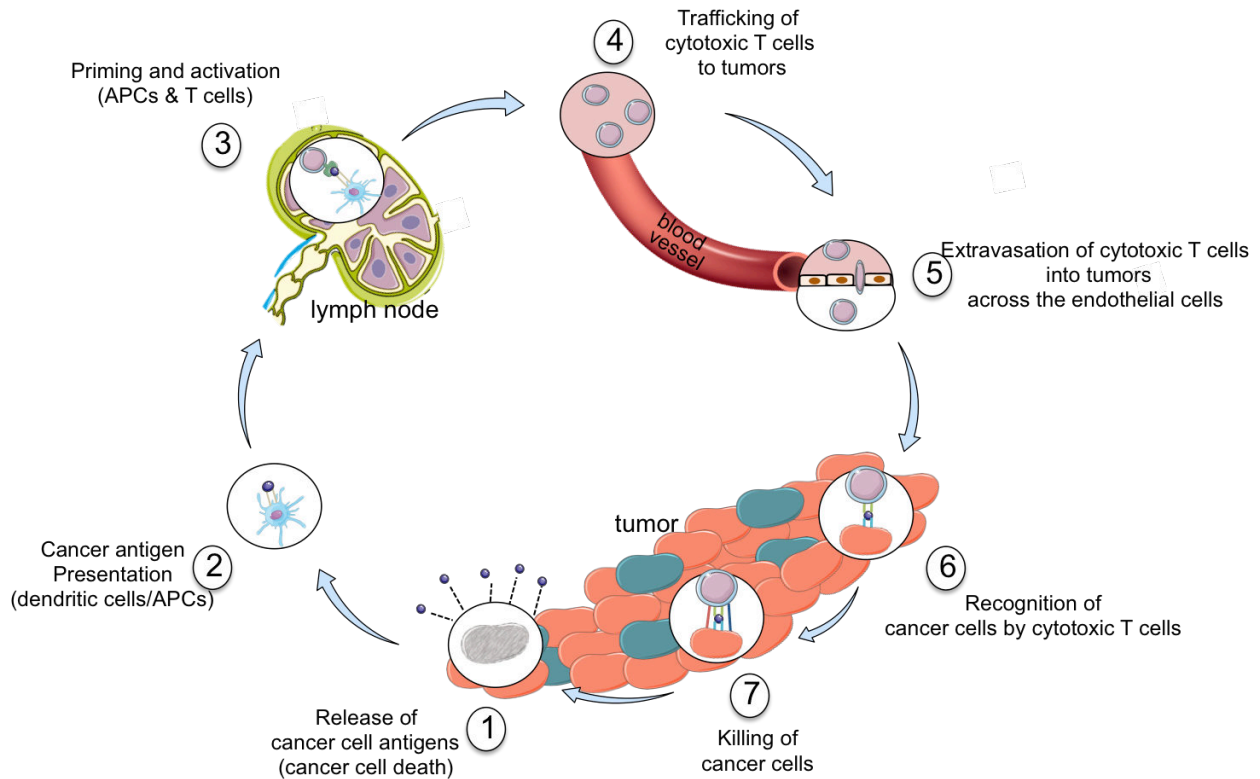
**Figure 5.** The concept of immunoediting adapted from (Dunn et al., 2002)



### 2.2.1. Anti-tumor immune response

During the elimination phase, the generation of an effective immune response against tumor cells involves a series of stepwise events, so-called cancer-immunity cycle illustrated in Figure 6 (Chen and Mellman et al., 2013).

**Figure 6.** The Cancer immunity cycle adapted from (Chen and Mellman et al., 2013)



This cycle can be divided into seven major steps: neoantigens are released from tumor cells (Step 1), and captured by antigen-presenting cells (APCs) such as DC (Step 2) which migrate to lymph nodes to prime and activate T cells (Step 3). Primed anti-tumor T cells migrate to the tumor site via the blood circulation (Step 4) and infiltrate tumors (Step 5) where they recognize tumor cells (Step 6) and kill them (Step 7).

The antitumor immune response is largely dependent on activation of **tumor-specific cytotoxic CD8<sup>+</sup> T cells** (Boon et al., 1994). Tumor-infiltrating CD8<sup>+</sup> T cells are frequently associated with

favorable prognostic in a large number of cancers (Pagès et al., 2005). The success of novel immunotherapies targeting immune checkpoint that restore T cell function in cancer patients also demonstrate the primary role of tumor-specific CD8<sup>+</sup> T cells (Pardoll et al., 2012). The activation of this response initiates mainly from endocytosis of dead neoplastic cells potentially containing tumor-associated antigens by antigen presenting cells (APC) including macrophages, conventional dendritic cells (cDC) and B cells (step 1). They uptake, process and present (step 2) antigens to prime naïve CD4<sup>+</sup> T cells into Th1 CD4<sup>+</sup> T cells and naïve CD8<sup>+</sup> T cell into cytotoxic CD8<sup>+</sup> T cells in the lymph node (step 3). cDC are divided into two main lineages: cDC1 and cDC2 (Guilliams et al., 2014, 2016; Mildner and Jung et al., 2014). cDC1 are the APC that carries ability to cross-present exogenous antigen in MHCI context and activate CD8<sup>+</sup> T cells. The activated CD8<sup>+</sup> T cells migrate into tumors (step 4 and 5) where they recognize and kill tumor cells in an antigen specific manner (step 6 and 7). These cytotoxic CD8<sup>+</sup> T cells are supported by IFN- $\gamma$  secreted by Th1 CD4<sup>+</sup> T cells, which are themselves mutually supported by classically activated pro-inflammatory macrophages. This cross-presentation by cDC1 is well established to be necessary for inducing a protective cytotoxic CD8<sup>+</sup> T-cell response (Hildner et al., 2008). cDC2, on the other hand, is a heterogeneous population and displays enhanced MHCII antigen presentation and preferentially activates CD4<sup>+</sup> T cells. They can secrete a large variety of cytokines and can induce the differentiation of Th1, Th2 and Th17 (Blasio et al., 2016; Nizzoli et al., 2016; Sittig et al., 2016). Yet, their role in tumor immunity is less studied.

Besides the adaptive immune responses, cellular actors of the innate immunity also contribute to the anti-tumor immune responses. They include **Natural killer (NK), NKT and  $\gamma\delta$  T cells** but also **macrophages** and **neutrophils**. As described above, effective tumor cell killing by cytotoxic CD8<sup>+</sup> T cells requires the presence of the tumor antigen presented in MHCI molecule on the surface of the cancer cells. During malignant transformation, some cancer cells may lose the expression of MHCI molecule on their cell surface to avoid the killing by CD8<sup>+</sup> T cells. In this case, their elimination is eventually executed by **NK cells**. NK cell cytotoxicity is controlled by a variety of inhibitory and activating receptors (Cerwenka and Lanier et al., 2001). The inhibitory receptors bind MHCI, therefore, the lack of MHCI on malignant cells renders them susceptible

to killing by NK cells. In addition, up-regulation of ligands of activating receptors such as NKG2D ligands on the surface of tumor cells can also trigger killing by NK cells (Cerwenka et al., 2001; Diefenbach et al., 2001; Raulet et al., 2003). An alternative mechanism of killing of tumor cells by NK cells can occur by an antibody-dependent cellular cytotoxicity (ADCC) mechanism where the Fab portion of an IgG antibody binds to the surface of target and bridges to an Fc receptor on the NK cells (Wu and Lanier et al., 2003). A role for NK cells in anti-tumor immunity is supported by several studies that linked the presence of NK cells in tumor with a favorable disease prognostic (Agarwal et al., 2016; Pasero et al., 2015; Villegas et al., 2002).

Besides NK cells, two types of T cells also play a role. **NKT cells** are defined as a type of innate lymphocytes that express both T cell and NK cell markers. Their abundance in cancer patients and in mouse cancer models is of importance in mediating protection against tumors. They can perform direct lysis of tumor targets, engaging the T cell receptor (TCR) but also NK receptors on their surface binding ligands induced by stress on tumor cells. This has been demonstrated for NKG2D. (Altman et al., 2015).

**$\gamma\delta$  T cells** are a small group of T cells that express distinct TCR  $\gamma$  and  $\delta$  chains. They have also been implicated in the direct killing of tumors using similar mechanisms as NK cells such as perforin/granzyme, TRAIL and FasL, ADCC (Zhao et al., 2018).

Besides cytotoxicity, NK, NKT and  $\gamma\delta$  T cells are an early source of IFN- $\gamma$  and TNF- $\alpha$  that contributes to the development of protective adaptive T cell responses.

Lastly, **macrophages and neutrophils** can participate to the anti-tumor immune responses. However, a body of studies suggested a dual role of both cell types in response to tumor (Fridlender et al., 2009; Martinez et al., 2008; Mills et al., 2000). Those controversial roles and functions will be described in detail in the later chapter in this manuscript. They have been named “classically-activated macrophages” (or M1) and tumor-associated neutrophils (TAN) N1, which most likely contribute to anti-tumor immunity, by opposition to “alternatively-activated macrophages” (or M2 and M2-like) and TAN N2, which are involved in pro-tumor immunity.

## 2.2.2. Pro-tumor immune response and immunosuppression

The interactions between tumor cells and infiltrating immune cells evolve overtime and are associated with **chronic inflammation and a shift from Th1 to Th2 immune responses** which favor tumor growth. The tumor microenvironment and tumor cells can corrupt recruited immune cells such as **macrophages and neutrophils** which shift to a pro-tumoral phenotype. In addition, several mechanisms of immunosuppression are put in place. They involve **regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and immune checkpoints**. Altogether, these mechanisms suppress cytotoxic anti-tumor immune responses and favor a pro-tumoral microenvironment.

### ➤ Deviation from Th1 to Th2 inflammatory response

**Deviation from Th1 to Th2 inflammatory response** is believed to facilitate tumor promotion (Mantovani et al., 2008). This was demonstrated by the fact that DCs, after uptake of released tumor antigens, are conditioned by the thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, secreted by cancer associated fibroblast (CAFs) and migrate to draining LNs to activate tumor-antigen specific CD4<sup>+</sup> T cells toward a Th2 phenotype (Protti and De Monte et al., 2012). The Th2 cytokines IL-4 and IL-13 participate to the polarization of anti-inflammatory macrophages (classically activated or M1) into **pro-inflammatory macrophages (alternatively activated or M2)** which produce IL-10, favoring immune suppression (Martinez and Gordon, 2014). Similarly, TGF- $\beta$  in the tumor microenvironment favor the development of pro-tumoral neutrophils N2 (Fridlender et al., 2009). Both **tumor-associated macrophages (TAM) M2 and tumor-associated neutrophils (TAN) N2** have been implicated in the promotion of tumor cell growth through various mechanisms such as angiogenesis and ECM remodeling, promotion of tumor growth, suppression of an anti-tumor immunity. More information on TAN is provided in the next section describing their anti-tumoral and pro-tumoral functions.

### ➤ Immunosuppression

**Tregs** have a prominent role in maintaining peripheral tolerance and immune suppression. The first evidence of an increase of Tregs at the tumor site was reported in non-small-cell lung and

ovarian cancers in 2001 (Woo et al., 2001). The observation was extended to many other cancers such as ovarian, breast, colorectal, lung, pancreatic cancers and melanoma (Zou et al., 2006). The high infiltration of Tregs correlates with a poor prognosis in many types of cancer including in breast cancer (Bates et al., 2006), gastric cancer (Sasada et al., 2003), and ovarian cancer (Curiel et al., 2004). They are mainly recruited into tumors in response to chemokines secreted by both tumor cells and innate immune cells including CCL-22, CCL-17, CCL-28 (Chaudhary and Elkord et al., 2016). Two types of Tregs have been described: natural Treg (nTregs) are derived from FoxP3<sup>+</sup> precursors in the thymus and inducible Treg (iTregs) which upregulate FoxP3 are derived from naïve CD4<sup>+</sup> T cell precursors (Fontenot et al., 2003; Hori et al., 2003). They are both found in tumors. Tregs functionally suppress immune responses either by contact-dependent and independent mechanisms. The most prevalent mechanism is the secretion of immunosuppressive cytokines TGF- $\beta$  (Transforming growth factor  $\beta$ ), IL-10 and IL-35 which suppress T cell anti-tumor proliferation and effector functions (Vignali et al., 2008). Indirectly, Tregs are able to suppress DC maturation, priming them towards a tolerogenic phenotype and impairing their ability to activate effector T cells and CTLs, resulting in T cell exhaustion. Moreover, they can condition DCs, through interaction between cytotoxic T lymphocyte antigen-4 (CTLA4) on Tregs and CD80/CD86 on DC, to express indoleamine 2,3-dioxygenase (IDO). This later is a potent regulatory molecule that induces the catabolism of tryptophan into pro-apoptotic metabolites that results in T cell anergy and the suppression of effector T cells (Fallarino et al., 2003).. By expressing the high affinity IL-2 receptor chain (CD25), Tregs can also locally deplete IL-2 from the microenvironment, leading to apoptosis of effector T cells. Lastly, Tregs can induce a direct cytolysis of effector T cells by a mechanism that is mediated by granzymes and perforins (Gondek et al., 2005). Similarly, Tregs suppress anti-tumor NKT cells (Azuma et al., 2003)

Recently, a body of studies highlighted **myeloid-derived suppressor cells (MDSCs)** as one of the main driver of an immunosuppressive tumor microenvironment (Bronte et al., 2016; Gabrilovich et al., 2017). They represent a heterogeneous population of immature myeloid cells (IMC) that fail to terminally differentiate and exhibit a strong capacity to suppress the functions

of T and NK cells (Gabrilovich and Nagaraj et al., 2009). It is thought that because an emergency granulopoiesis is triggered by peripheral inflammation such as cancer, undifferentiated cells can be released from the bone marrow in the circulation (Youn et al., 2012). MDSCs are divided into two groups of cells: monocytic MDSCs (M-MDSCs) and granulocytic or polymorphonuclear MDSCs (PMN-MDSCs). M-MDSCs are phenotypically and morphologically similar to monocytes, whereas PMN-MDSCs are similar to neutrophils.

In mice, M-MDSCs are defined as  $CD11b^+Ly6G^-Ly6C^{high}$  cells which is the classic phenotype of inflammatory monocytes present in healthy mice. But, they do not express CD11c, neither MHCII molecule, and can be readily distinguish from TAMs expressing high expression of F4/80 and low to intermediate expression of Ly6C. PMN-MDSCs, on the other hand, can be defined as  $CD11b^+Ly6G^+Ly6C^{low}$ , which is a typical phenotype for neutrophils (Bronte et al., 2016; Damuzzo et al., 2015).

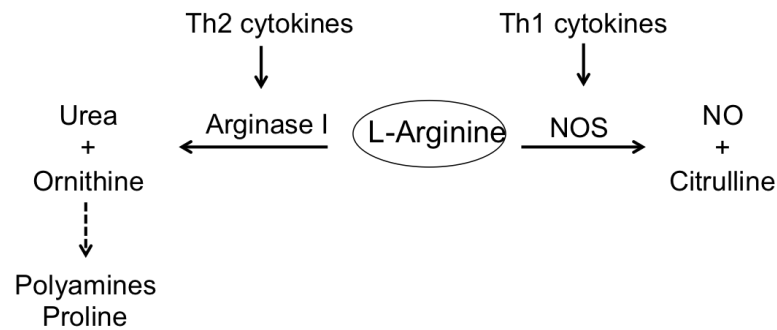
In humans, M-MDSCs have the phenotype  $CD11b^+CD14^+CD15^-CD33^+HLA-DR^{-/low}$ , and can be distinguished from monocytes expressing  $HLA-DR^+$  (Bronte et al., 2016; Damuzzo et al., 2015). PMN-MDSCs and neutrophils share a similar phenotype:  $CD11b^+CD14^-CD15^+$  (or  $CD66b^+$ )  $CD33^+$ . However, their different densities allow their discrimination. Following density gradient of human whole blood, PMN-MDSCs has been found co-purified with the fraction of peripheral blood mononuclear cells. In contrast, neutrophils are isolated from the high-density fraction (Sagiv et al., 2015).

In tumors, the identity of MDSCs remains unclear. They differ phenotypically from TAM but cannot be distinguished from TAN. MDSCs were originally defined by their function of immunosuppression but other tumor-promoting functions may also be attributed to them (Groth et al., 2018).

Several mechanisms of immunosuppression have been described, one of which involves the metabolism of L-arginine (simplified in the Figure 7). This metabolism is central to immunosuppression of MDSC. L-arginine can be metabolized by multiple isoforms of nitric oxide synthases (NOS1, NOS2 and NOS3) into citrulline and nitric oxide (NO), this latter is a crucial mediator in cytotoxic mechanism and vasodilation (Hibbs et al., 1987). Alternatively, L-

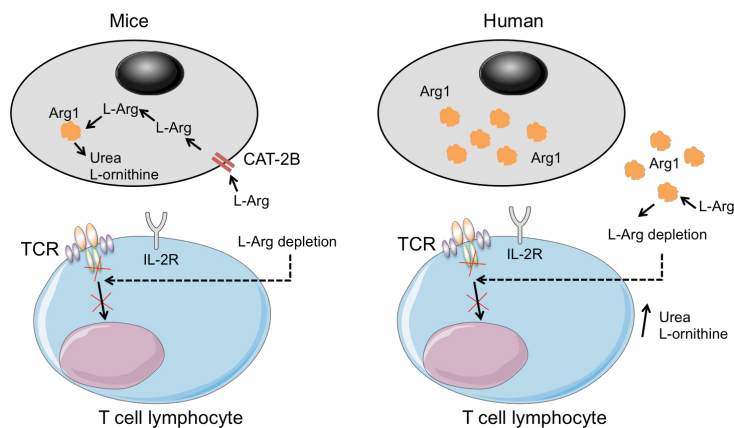
arginine is metabolized by arginases (I and II) into urea and L-ornithine, this latter is the precursor for the production of polyamines essential for cell proliferation (Wu and Morris, 1998).

**Figure 7.** A simplified metabolism network of L-arginine adapted from (Raber et al., 2012)



Degradation of L-arginine by arginase I either secreted in the microenvironment (human MDSCs) or present inside the cells (mice MDSCs) leads to a decrease of extracellular level of L-arginine. This reduction deprives T cells from L-arginine, thus inhibiting their proliferation (Figure.8) (Raber et al., 2012; Rodriguez et al., 2004).

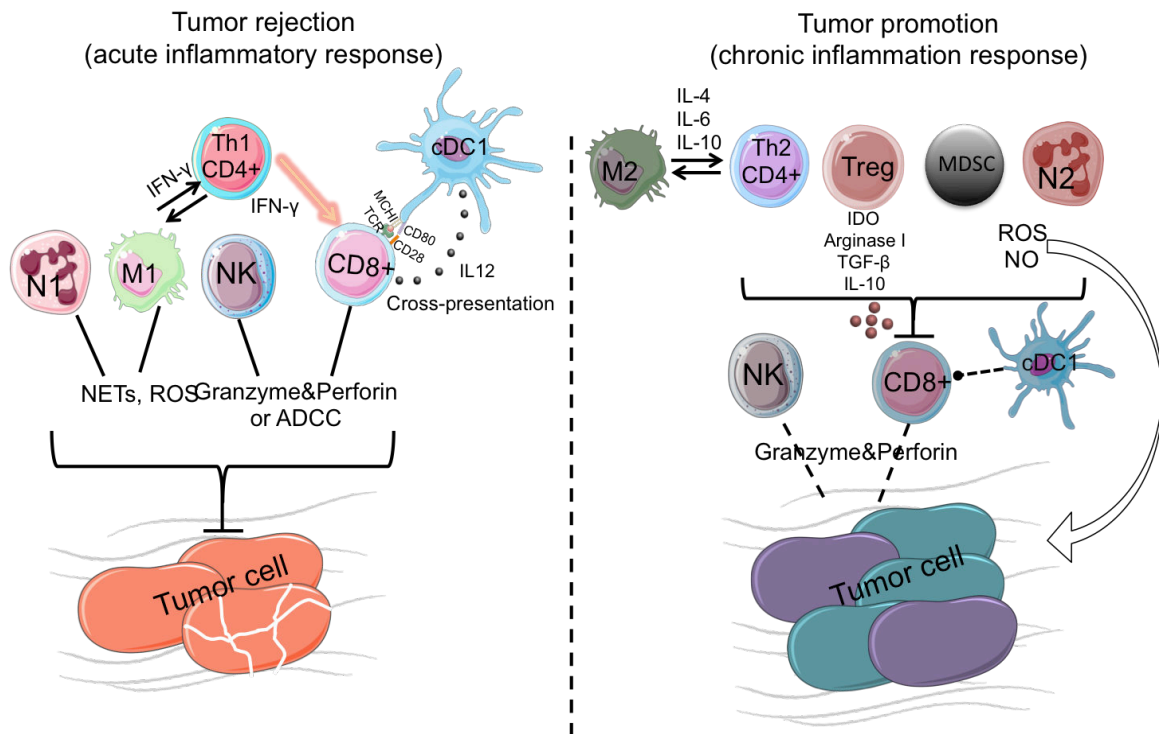
**Figure 8.** Inhibition of T cell function induced by arginase I adapted from (Raber et al., 2012)



The expression of arginase I and NOS can be differentially regulated by Th1 and Th2 cytokines respectively (Hesse et al., 2001). M-MDSCs and PMN-MDSCs have been reported to use distinct preferential pathways to metabolize L-arginine. Tumor-associated M-MDSCs utilize NOS2 to metabolize L-arginine into nitric oxide (Youn et al., 2008), whereas PMN-MDSCs are the major source of arginase I in tumor-bearing mice (Rodriguez et al., 2004).

In addition to their inhibitory action on T cell proliferation, MDSCs produce reactive oxygen and nitrogen species (ROS and RNS) that can have direct toxic effect on anti-tumoral T cells (Liu et al., 2015; Raber et al., 2014). NO produced by MDSCs was also reported to mediate a blocking of the homing of T cells by interfering with cell adhesion molecules CD62L and CD44 (Schoupe et al., 2013). MDSCs can induce the generation of Treg and TAM M2 in an IL-10 and IFN- $\gamma$  mediated way (Huang et al., 2006). MDSCs up-regulate ectoenzymes CD39 and CD73 that generate adenosine from ATP, decreasing T cell functions (Li et al., 2017). And lastly, MDSCs up-regulate PD-L1, the ligand of the inhibitory receptor PD-1 expressed by T cells as a mechanism of retro control (Lu et al., 2016; Yamauchi et al., 2018). This interaction is one of the **immune checkpoints** recently described that can induce T cell anergy and apoptosis. Anti-tumoral T cells are regulated by a variety of co-stimulatory and inhibiting signals delivered by cell surface receptors. They are called immune checkpoints. Their function is to maintain self-tolerance but in cancer patients, their expression can be deregulated leading to the immunosuppression of the anti-tumor response. The development of antibodies that block immune checkpoints is a revolution in the field of tumor immunotherapies and Prof J. Allison and T. Honjo won the Nobel price of medicine 2018 for their discovery on CTLA4 and PD1 targeting (Freeman et al., 2000; Leach et al., 1996).

**Figure 9.** Dual role of immune system “Anti-tumor and Pro-tumor immune response” adapted from (Denardo and Coussens et al., 2007).



### 3. Neutrophil biology

#### 3.1. Development

**Hematopoiesis** is the process by which blood cells are formed. Before birth, it happens in the yolk sac of the embryo and subsequently in the liver, thymus and spleen. But, in adult the development mainly occurs in the bone marrow.

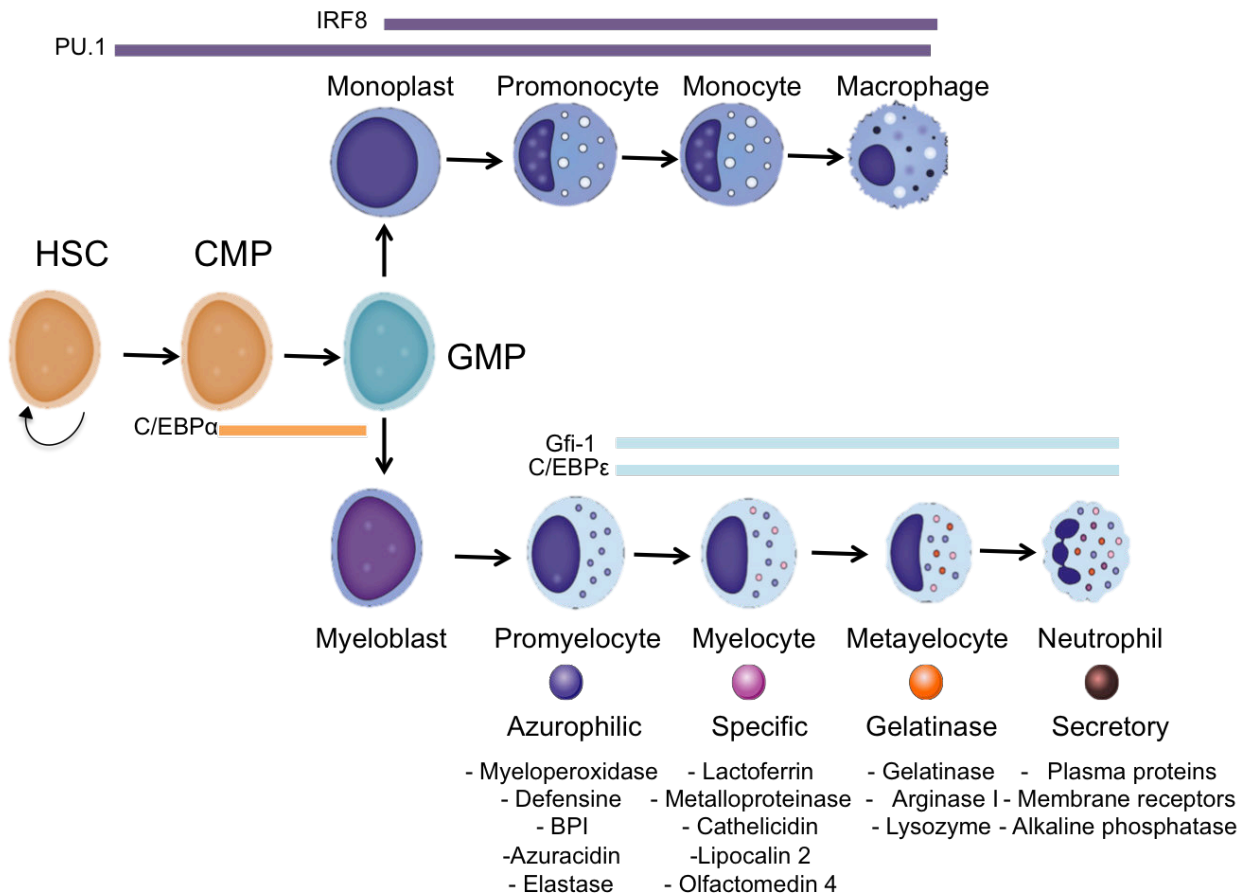
Neutrophils, like other types of blood cells, develop from pluripotent hematopoietic stem cells (HSCs) by getting through an orderly unidirectional progression of proliferation, differentiation and maturation (Bjerregaard et al., 2003; Lawrence et al., 2018; Rosenbauer and Tenen et al., 2007). Their development is a highly controlled staged process, summarized in Figure 10. It is divided into two sequential stages: multiplication and maturation. HSCs differentiate into highly

proliferative hematopoietic progenitor cells (HPCs), which are committed to specific cell lineage. Among them, the common myeloid progenitor (CMP) differentiates into a granulocyte monocyte progenitor (GMP) giving rise to neutrophils, eosinophils, monocytes and macrophages. This differentiation is under the control of specific transcription factors. The transcription factor PU.1 is required to generate CMP from HSCs and C/EBP $\alpha$  is required to generate GMP from CMP. Then, while PU.1 is still expressed, C/EBP $\alpha$  is lost in later stages of differentiation. GMP under the control of Irf8 differentiates into monocytes and macrophages while both Gfi-1 and C/EBP $\epsilon$  are required to give rise to neutrophils and eosinophils. It is the lack of GATA-1 that drives them to differentiate into neutrophils (Lawrence et al., 2018; Rosenbauer and Tenen, 2007). The different consecutive stages of maturation leading to the generation of mature neutrophils include myeloblasts, promyelocytes, myelocytes, metamyelocytes and neutrophils. Historically, they were differentiated by the shape of their nucleus with myeloblasts and promyelocytes having a large and round nucleus, myelocytes and metamyelocytes, a kidney-shaped nucleus and then a band-like nucleus and neutrophils a segmented nucleus. Maturation depends on cytokines and growth factors, the most prominent being G-CSF and others playing a redundant role such as GM-CSF, IL-6 and KIT ligand (Bhattacharya et al., 2015; Gregory and Houghton, 2011; Liu et al., 1997; Molineux et al., 1991; Petit et al., 2002). Also, IL-23 secreted by phagocytes and IL-1 $\beta$  released from dying cells induces the production of IL-17 by T helper 17 (Th17) cells, leading to G-CSF expression, which enhances neutrophil differentiation (Stark et al., 2005; Ueda et al., 2009).

**Granulopoiesis** or granule formation occurs sequentially during neutrophil development (Lawrence et al., 2018). Four types of granules and secretory vesicles are acquired throughout neutrophil maturation. Granulopoiesis is initiated with the appearance of **primary granules** (or **azurophilic granules** according to their affinity for the basic dye azure) in myeloblasts and promyelocytes. These primary granules contain high amount of myeloperoxidase (MPO) participating to the oxidative burst and toxic molecules such as lysozyme, defensins, serine proteases including elastase (NE), cathepsin G and proteinase 3 (PR3). The presence of MPO is limited to the transition between promyelocytes and myelocytes. Then, **secondary (or specific)**

**granules** defined by a high content of lactoferrin and a low content of gelatinase are generated in myelocytes and metamyelocytes. They contain anti-microbial compounds such as neutrophil gelatinase-associated lipocalin (NGAL) and human cathelicidin-18 (hCAP-18). **Tertiary (or gelatinase) granules**, in contrast, contain a low amount of lactoferrin and a high amount of gelatinase (MMP9) and arginase 1. Finally, **secretory vesicles** are formed in segmented neutrophils and they contain plasma proteins, suggesting that they are formed by endocytosis (Amulic et al., 2012; Faurschou and Borregaard et al., 2003; Lawrence et al., 2018).

**Figure 10.** Neutrophil development showing different cellular stages with key transcription factors and granule components adapted from Lawrence et al., 2018 and Rosenbauer and Tenen et al., 2007



## **3.2. Migration/circulation of neutrophils**

### **3.2.1. Retention of neutrophils in the bone marrow**

Under physiological conditions, mature neutrophils are mainly found in the bone marrow and small pools are also found in lung, spleen and liver. Mature neutrophils that constitute the bone marrow reserve localize in hematopoietic cords, which are separated from the blood stream by the bone marrow sinusoidal endothelium. They are retained in there by the interaction between CXCR4 on the surface of neutrophils and chemokine SDF-1 $\alpha$  (CXCL-12) expressed by bone marrow stromal cells and osteoblasts (Martin et al., 2003). The loss of CXCR4 but also the acquisition of CXCR2 provides signals for their release in the circulation (Eash et al., 2010). G-CSF is a major signal that trigger release of neutrophils in the circulation. It induces the production of CXCR2 ligands (KC, MIP-2) and activates neutrophils that release NE and cathepsin G which degrades CXCL-12. CXCR4 is also endocytosed and degraded (Köhler et al., 2011; Petit et al., 2002).

Like previously described, during cancer progression, myelopoiesis can be altered leading to a dramatic accumulation of MDSCs comprising M-MDSCs and PMN-MDSCs. Different molecules have been involved in the regulation of this process such as retinoblastoma protein (Rb), a tumor suppressor protein, (Youn et al., 2013), RORC1 master regulator (Strauss et al., 2015).

Recently, Travelli et al reported a new role of nicotinamide phosphoribosyltransferase (NAMPT) in mobilization of MDSCs (Travelli et al., 2019). NAMPT is the limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis, carrying an important role in metabolism and inflammation. It has been observed to be overexpressed in many types of tumors and this aberrant expression was associated with tumor progression (Lucena-Cacace and Carnero et al., 2018). The recent study revealed that NAMPT could inactivate the induction of CXCR4 gene expression by HIF-1 $\alpha$ , resulting in disruption of the CXCR4 retention axis of MDSCs in bone marrow, thus enhancing their release into periphery.

### 3.2.2. Recruitment to inflamed tissues: Extravasation (postcapillary venules)

Extravasation is the common cascade of neutrophil (and also monocytes) migration out of blood vessels that involves four consecutive steps: **rolling, adhesion, crawling and transmigration**. In normal condition, leukocytes freely flow in the blood stream. **Extravasation** is initiated by the changes on the surface of endothelium, resulting from stimulation by inflammatory mediators (Histamine, thrombin, leukotriene, lipopolysaccharides, oxidant...). These signals are generally released consecutively to an intrusion by pathogens either by tissue resident sentinel leukocytes or by endothelial cells expressing pattern-recognition receptor (PRR). When activated, endothelium P-selectin, which is an adhesion molecule pre-synthesized and stored in Weibel-Palade bodies, is mobilized to the membrane and expressed on the surface. P-selectin is a very long molecule with nine consensus repeats. This structure is essential to its function to reach out and grab leukocytes, mediating the **initial tethering** between leukocytes and endothelium followed by **ROLLING STEP** (Patel et al., 1995). When blocking P-selectin, the early recruitment is totally affected during an inflammatory response (Dore et al., 1993). However, this impairment is rescued within minutes by a second selectin, *de novo* synthesized molecule which is E-selectin (Bevilacqua et al., 1987). In normal condition, E-selectin function overlaps with P-selectin, temporally helping to enhance leukocyte recruitment. P-selectin and E-selectin bind to their glycosylated ligands, P-selectin glycoprotein ligands 1 (PSGL1) that allow neutrophils to adhere reversibly to the vessel wall (other surface molecules of neutrophils and of endothelium are listed in (TABLE 1). Moreover, L-selectin which is known to be mainly involved in homing of lymphocytes to lymph node, is found to be expressed by circulating neutrophils and might facilitate a secondary tethering to an already rolling neutrophils. The rolling has an important function to increase the contact of neutrophils with the chemokines, which are present on the endothelium. Those chemoattractant molecules are positively charged and immobilized on endothelium by binding to negatively charged heparan sulphates, which serve as anchors, preventing the shear forces of blood flow from washing these molecules away (Massena et al., 2010). This allows the formation of intravascular chemotactic gradients. Neutrophil chemotaxis involves CXCL-8 (or IL-8) in human

and its analogue CXCL1/KC, CXCL-2/MIP-2, and CXCL-5; CXCL-6/LIX in mice, which signal via CXCR-1 and CXCR-2 to activate neutrophils and subsequently promote their **ADHESION** to endothelium. The pro-inflammatory IL-17 was shown to regulate the expression of IL-8. Adhesion involves a change of conformation of the integrins LFA-1 (also known as  $\alpha 1\beta 2$ ;  $\beta 2$  integrin CD11a complexed with CD18) and MAC1 (also known as  $\alpha M\beta 2$ ; CD11b-CD18) expressed by activated neutrophils that can then bind to the immunoglobulin-like cell adhesion molecules 1 and 2 (ICAM-1, ICAM-2). This interaction triggers arrest and firm adhesion. Of note, a clear difference in function of both integrins was demonstrated by Phillipson et al., 2006. LFA-1 binding to ICAM-1 is essential to create a firm **ADHESION** of neutrophils to endothelium, and MAC1 is required for the **CRAWLING**. Indeed, neutrophils were found to initiate some crawling behaviors that allow them to transmigrate at a different location than the one where they adhere.

**TRANSMIGRATION** is a process in which neutrophils leave the blood vessels either by crossing the endothelial cell layer para-cellularly (between endothelial cells) or trans-cellularly (through an endothelial cells). This involves interaction between leukocyte integrins (LFA-1 and Mac-1) and ICAMs (ICAM-1, ICAM-2) as well as multiple different junction proteins (TABLE 1). The movement across the vessel wall is known as diapedesis. The majority of neutrophil transmigration occurs via junctions between adjacent endothelial cells (paracellular route) and only a minority happens through the bodies of endothelial cells (transcellular route) (Kolaczowska and Kubes, 2013). Then, they need to pass through basal membrane by partially digesting this later with their proteases. Finally, they migrate between pericytes, which are wrapped around blood vessels.

#### ➤ **Extravasation in lymph node**

Recent data suggested that neutrophils following various stimulations can be found in the draining lymph node (Hampton and Chtanova, 2016). They are recruited via blood vessels or afferent lymphatic (Beauvillain et al., 2011; Brackett et al., 2013; Gorlino et al., 2014). This recruitment required expression of CCR7 on the surface of neutrophils in response to CCL-19 and CCL-21. Indeed, neutrophils from *ccr7<sup>-/-</sup>* mice failed to migrate to the draining lymph nodes

(Beauvillain et al., 2011). Neutrophils also migrate to lymph node in response to CXCL-12, IL-17, CXCL-2 and IL-1 $\beta$  (Brackett et al., 2013; Gorlino et al., 2014).

➤ **Extravasation in lung and liver**

In some cases, the extravasation in microcapillaries can occur in a selectin-independent manner. The lung is fulfilled with mature, terminally differentiated neutrophils (Sibille and Marchandise, 1993; Peters, 1998). The concept is confirmed by direct evidence obtained from real-time neutrophil trafficking in the lung vasculature using intra-vital microscopy (Kreisel et al., 2010).

**Table 1.** Adhesion molecules involved in different stages of the neutrophil recruitment cascades in postcapillary venules, adapted from Kolaczowska and Kubes et al., 2013

Molecule on endothelium	Molecules on neutrophils
<b><i>Tethering and rolling</i></b>	
P-selectin	PSGL1 (positively regulates recruitment). PTX3 (negatively regulates recruitment)
E-selectin	PSGL1 . ESL1.CD44
PSGL1. GlyCAM (on rolling neutrophils)	L-selectin (on circulating neutrophils)
<b><i>Slow rolling</i></b>	
ICAM1	LFA1 (PSGL1-induced)
E-selectin	PSGL1.ESL1.CD44
<b><i>Arrest and adhesion</i></b>	
ICAM1	LFA1
VCAM1	VLA1
<b><i>Crawling</i></b>	
ICAM1	MAC1
<b><i>Transmigration and diapedesis</i></b>	
ICAM1.ICAM2	LFA1.MAC1
VCAM1	VLA4
CD99	CD99
PECAM1	PECAM1
JAMA	LFA1.JAMA?
JAMB	VLA4
JAMC	MAC1
CD99L2	?
VE-cadherin (negatively regulates recruitment). ESAM	Between endothelial cells

### **3.2.3. Reverse transmigration**

It is believed that during inflammation most of the neutrophils that extravagate from the blood into the site of inflammation die and are cleared by macrophages after they fulfill their functions (Savill et al., 1989; Silva et al., 2011). In addition, neutrophils are capable of reverse transmigration. Mathias et al. reported for the first time *in vivo*, that in zebrafish embryos, under sterile inflammatory conditions, extravagated neutrophils could subsequently re-enter the vasculature (Mathias et al., 2006). Recently, similar observation was also documented in mice (Woodfin, Nat Immunol et al., 2011). A mechanism was described involving leukotriene B<sub>4</sub> (LTB<sub>4</sub>) that stimulate proteolytic cleavage of endothelial JAM-C by neutrophil elastase (Colom et al., 2015). Reverse transmigration may be a part of the resolution of inflammation to avoid harmful effect of activated neutrophils on intact tissue.

## **3.3. Survival and apoptosis**

### **3.3.1. Neutrophil Life span**

For many years, neutrophils had been believed to have a short half-life of about 1.5 hours in mice and 8h in human (Roos, 1998). Recently, Pillay et al introduced a revolutionary concept of extremely long-lived neutrophils, up to 5.4 days for human and up to 12.5 h days for mice (Pillay et al., 2010). Using a novel technique to measure *in vivo* the half-life of neutrophils, the study does not require ex vivo cell isolation. Pillay and colleagues labeled neutrophils by administrating <sup>2</sup>H<sub>2</sub>O in drinking water. A mathematical model was used to estimate the half-life, with the assumption that the rates of neutrophils, which enter and leave the bloodstream, are equal. However, Paul S. Tofts raised many objections to Pillay's mathematical model (Tofts et al., 2011) by stating that <sup>2</sup>H<sub>2</sub>O is very likely to label also bone marrow neutrophils, thus leading to an overestimating of the lifespan of neutrophils in blood. The current belief is that the half-life is short in steady-state but can be longer in tissue, even up to several days. Inflammatory signals can increase the half-life of neutrophils (Haslett et al., 1991; Maiani et al., 2004). Then once, their function is no longer required, aged neutrophils die by constitutive (or spontaneous)

apoptosis. Under steady-state condition, aged neutrophils are removed from the circulation by mainly homing back to bone marrow in a CXCR4-dependent manner, where they are eliminated in order to maintain cell homeostasis (Furze and Rankin et al., 2008; Suratt et al., 2001).

### **3.3.2. Apoptosis**

Apoptosis is a conserved mechanism of programmed cell death. Two main apoptotic pathways have been described: an intrinsic and an extrinsic pathway. Neutrophils are able to undergo spontaneous apoptosis, through an intrinsic process involving many molecules from mitochondria, members of the Bcl2 family and caspases. Neutrophils constitutively express anti-apoptotic Mcl1 and BCL2A1, which are key Bcl2-family proteins. As they become senescent, the level of Mcl1 and BCL2A1 gradually decreases (Leuenroth et al., 2000). This degradation leads to activation of caspases 8 and 9 and further caspase-3. Bcl-associated X protein (BAX), another member of Bcl2-family, is pro-apoptotic. BAX is translocated in the mitochondria where it is cleaved to small fragments, which are unable to interact with other members of Bcl2 family, thus promoting apoptosis. In addition, ROS and oxidative stress can also lead to neutrophil apoptosis. They disrupt mitochondria transmembrane potential resulting in release of cytochrome c that will be associated with protease-activating factor 1 (APAF-1) to initiate apoptosis (Kennedy and DeLeo, 2009).

In contrast to spontaneous apoptosis, another type of apoptosis is induced by extrinsic factors such as FAS ligand, TNF $\alpha$  and TRAIL. These molecules bind to their receptors present on the surface of neutrophil and trigger apoptosis via a caspase-dependent or caspase-independent pathway (Kennedy and DeLeo, 2009).

### **3.3.3. Metabolism**

Maintaining a bioenergetic homeostasis and cell survival requires a critical level of adenosine triphosphate (ATP). For mammalian cells, glucose is main source of energy. It can be metabolized via two integrated metabolic pathways, glycolysis and oxidative phosphorylation, that efficiently convert glucose into ATP. Neutrophils are largely dependent on glycolysis (Borregaard and Herlin et al., 1982). Glycolysis consists of the conversion of glucose into

pyruvate through a series of enzyme that are present in the cytosol. This series of reactions generates two molecules of ATP. Pyruvate is next transported into the mitochondria and further metabolized to CO<sub>2</sub> by the Krebs cycle, which initiates oxidative phosphorylation and the translocation of protons across the mitochondrial inner membrane. This produces a proton gradient that drives the enzyme ATP synthase to convert adenosine diphosphate (ADP) into ATP in a loop, which generates in total 34 ATP per molecule of glucose. In addition to this breakdown of glucose via glycolysis, cells have the ability to metabolize alternative substrates, such a lipids and glutamine.

Upon activation, immune cells tend to switch to aerobic glycolysis which is less efficient but more rapid than oxidative phosphorylation. Aerobic glycolysis supports biosynthetic processes of the cell as it allows the uptake of larger amounts of glucose and the maintenance of elevated glycolytic flux. Glycolytic intermediates are then diverted into various pathways for the synthesis of biomolecules that support biosynthetic processes. For instance, glucose 6-phosphate (G6P), generated by the first step in glycolysis, can feed into the pentose phosphate pathway (PPP) to support nucleotide synthesis. This pathway also generates NADPH, a cofactor that is essential for various biosynthetic processes of pro-inflammatory molecules. Neutrophil effector functions require glucose metabolism, in particular it has been well described for the formation of neutrophil extracellular traps (Azevedo et al., 2015; McInturff et al., 2012; Rodríguez-Espinosa et al., 2015).

### **3.4. Function of neutrophils**

#### **3.4.1. Antimicrobial function**

Neutrophils are the first line defense and are recruited in high numbers at the sites of infections. They control infection by different mechanisms such as (1) **phagocytosis**, a process in which neutrophils engulf pathogens (2) **degranulation**, a process consisting in releasing mediators stored in the granules (3) **ROS production** that is toxic for pathogens and (4) **netosis**,

a process of extracellular projection of processed chromatin bound to granular and selected cytoplasmic proteins.

They express a diverse range of surface receptors in order to recognize microbial ligands. A powerful agonist is the N-formyl-L-methionyl-L-leucyl-L-phenylalanine peptide (fMLP), a bacterial product known to stimulate G protein-coupled receptors (GPCRs) on the surface of neutrophils, leading to activation of the intra-cytoplasmic subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Babior et al., 1984a). This NADPH oxidase catalyzes the oxidation of NADPH and nicotinamide adenine dinucleotide (NADH) coenzyme to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Babior et al., 1984b). This massive and rapid non-mitochondrial generation of ROS in the phagolysosome is known as **respiratory or oxidative burst** and is mainly responsible for the killing activity of neutrophils (Nordenfelt and Tapper et al., 2011).

Other surface receptors involved in microbial recognition include C-type lectins and Toll-like receptors (TLR). TLRs are characterized by their cellular localization and the PAMPs (pathogen-associated molecular patterns) and DAMPs (Damage-associated molecular patterns), which they recognize. They are expressed in many different types of innate immune cells. Human neutrophils express all TLRs except TLR3 (Hayashi et al., 2003). TLR and C-type lectins trigger neutrophil migration, phagocytosis, programmed cell death, ROS generation and cytokine production.

Neutrophils also use antimicrobial molecules such as MPO, neutrophil elastase (NE), cathepsin G, and defensins to combat infection. Similarly, another strategy is the release of NETs which are extracellular DNA composed of decondensed chromatin, histones and anti-microbial proteins and peptides. These structures are released from the nucleus and lead to apoptosis or survival of the neutrophil. NETs prevent infection spreading and are highly efficient at killing pathogens as they harbor a high concentration of the toxic proteins from azurophilic granules, including NE, cathepsin G, PR3, defensins and cathelicidin LL-37 (Brinkmann et al., 2004; Papayannopoulos et al., 2010; Pilsczek et al., 2010).

### 3.4.2. Function in cancer

Neutrophils infiltrate tumors, sometimes in high proportions, and it is associated with inflammation, one of a hallmarks of Cancer (Hanahan and Weinberg et al., 2000, 2011). An increased number of circulating neutrophils has been detected in cancer patients and tumors (Manz and Boettcher et al., 2014; Templeton et al., 2014). But contradictory roles for neutrophils in cancer progression have been reported. Indeed, the absence of neutrophils in tumor-bearing mice can either speed up or slow down cancer progression (Brandau et al., 2013; Piccard et al., 2012). Within the tumor microenvironment, many mechanisms have been proposed for both roles (Coffelt et al., 2016).

#### 3.4.2.1. Anti-tumoral activity

The anti-tumor activity of TAN is characterized by their cytotoxicity towards tumor cells but also by their stimulation of innate and adaptive anti-tumoral immune responses.

##### ➤ Cytotoxicity

TAN uses their antimicrobial arsenal to kill tumor cells in a similar way to pathogen killing. Upon activation, they degranulate and liberate cytotoxic molecules like ROS, proteases and defensins. They can also release NETs that contribute to tumor cell damage. Tumor cells die both by apoptosis and necrosis. Neutrophils express Fc receptors and trigger antibody-dependent cellular cytotoxicity (ADCC) (Stockmeyer et al., 2003). This mechanism is well demonstrated *in vitro* and *in vivo* in mice (Albanesi et al., 2013) but evidence for a role of neutrophil-mediated ADCC in IgG-based immunotherapies in human remains limited. Recently, Matlung et al and colleagues reported that neutrophil ADCC involves a specific mechanism called “trogoptosis”, related to trogocytosis, and different from the granule exocytosis-dependent mechanism used by NK cells and CD8<sup>+</sup> T cells or the phagocytic cell death used by macrophages (Matlung et al., 2018).

Granule exocytosis-dependent mechanism is also a mechanism of tumor cell killing used by TAN. The expression of granzymes by neutrophils has been highly controversial but Martin et al recently reported novel evidence for granzyme B-mediated neutrophil cytotoxicity (Martin et

al., 2018). They observed that granzyme B, is induced in anti-tumor neutrophils stimulated by TLR agonist treatment. These granzyme B<sup>+</sup> neutrophils are found to localize very closely to apoptotic tumor cells after treatment (Martin et al., 2018).

#### ➤ Immunostimulation

Neutrophils regulate adaptive immunity through multiple cross-talk with other immune cells (Mantovani et al., 2011). The activation of neutrophils can increase maturation of dendritic cells and as a result, T cell proliferation and Th1 polarization (Gisbergen et al., 2005; Megiovanni et al., 2006). Neutrophils can cross-present antigen to naïve CD8<sup>+</sup> T cells which then differentiate into cytotoxic CTL (Beauvillain et al., 2007). A few studies also reported that activated neutrophils can present antigen and provide co-stimulatory signals for T cell activation (Ashtekar and Saha et al., 2003; Radsak et al., 2000). Eruslanov et al reported that neutrophils isolated from human lung tumor and nonmalignant lung tissue in the early stage of the disease could stimulate T cell proliferation and release of IFN- $\gamma$  via up-regulation of co-stimulatory molecules such as CD54, CD86, OX40L, and 4-1BBL on neutrophil (Eruslanov et al., 2014a). They named them “APC-like hybrid TANs”, based on simultaneous expression of APC markers (CD14<sup>+</sup> HLA-DR<sup>+</sup>HLA-ABC<sup>high</sup>CCR7<sup>+</sup>CD86<sup>+</sup>CD206<sup>+</sup>) and neutrophil markers (CD11b<sup>+</sup>CD66b<sup>+</sup>CD15<sup>high</sup>). Functionally, these APC-like hybrid TANs could produce pro-inflammatory cytokines TNF- $\alpha$  and IL-12, phagocyte, and cross-present tumor antigens for activation of antitumor T-cell responses (Singhal et al., 2016). Governa V et al reported that CD66b<sup>+</sup> neutrophil infiltration in colorectal cancer was significantly associated with increased survival. These cells frequently co-localized with CD8<sup>+</sup> T cells and they could enhance *in vitro* CD8<sup>+</sup> T cell activation, proliferation and IFN- $\gamma$  secretion induced by suboptimal concentrations of anti-CD3 antibodies (Governa et al., 2017).

#### 3.4.2.2. Pro-tumoral activity

Besides anti-tumoral functions, TAN display pro-tumoral roles contributing to tumor initiation, growth, and metastasis (Brandau et al., 2013; Coffelt et al., 2016; Piccard et al., 2012). The mechanisms are discussed below.

### ➤ Tumor initiation

Inflammation is one of the hallmarks of Cancer implicated in tumor initiation and it is associated with recruitment of high numbers of neutrophils. This triggers **genotoxicity** in which TAN participates, notably through the production of ROS and RNS (Gungor et al., 2010). In the chemically (DMBA/TPA)-induced skin carcinogenesis or colitis-associated colon carcinoma, the impairment of neutrophil recruitment prevented tumor formation (Jamieson et al., 2012; Shang et al., 2012).

### ➤ Tumor proliferation and angiogenesis

A large number of studies have now shown that depletion of neutrophils *in vivo* decreases tumor growth and that high level of TAN infiltrating tumors associates with poor clinical outcome and advanced disease. TAN can promote tumor growth by participating to **angiogenesis and ECM remodeling**. Angiogenesis triggered by the microenvironment is a crucial component for tumor progression and metastasis (Folkman et al., 1971). This is the process of forming new blood vessels from existing ones and requires degradation of the vascular basement membrane and remodeling of the ECM to allow endothelial cells to migrate and invade into the surrounding tissue. TAN express matrix metalloproteases (MMPs), a family of enzymes that proteolytically degrade ECM components. They also secrete proangiogenic factors such as vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs), epidermal growth factor (EGF) that provide signals to initiate and sustain angiogenesis. These factors bind to their respective receptors on endothelial cells, the interaction inducing proliferation and/or migration of tumor cells (Li et al., 2003). However, to become active, they need to be cleaved by MMPs, in particular MMP-9, which has been described to be the main actor in angiogenesis (Bergers and Benjamin et al., 2003). TAN thus contribute to the process by providing the main source of MMP-9 (Deryugina et al., 2014). In addition to angiogenesis, TAN **promote tumor growth** through a variety of mechanisms. TAN were shown to limit cancer cell senescence (Di Mitri et al., 2014). They could participate to the recruitment of Th17 that contribute to tumor growth (Ortiz et al., 2015). TAN secrete growth factors such as Hepatocyte growth factor (HGF) that binds to its receptor cMET on tumor cells, triggering tumor growth

and metastasis but also angiogenesis (Wislez et al., 2003). TAN secrete the cytokine Oncostatin M implicated in triggering VEGF production, contributing to neutrophils promotion of tumor growth and angiogenesis (Lauber et al., 2015; Queen et al., 2005).

#### ➤ **Immunosuppression**

Many studies reported a role for TAN in suppressing T cell proliferation. Similarly to PMN-MDSC, TAN up-regulates arginase-1 which catalyzes the hydrolysis of arginine, an essential amino acid necessary in many cellular processes, such as the proliferation of T cells (Rodriguez et al., 2004; Rodríguez and Ochoa et al., 2008). TAN also up-regulates iNOS that convert L-arginine into NO and L-citrulline, thus contributing to arginine deprivation from the local environment (Coffelt et al., 2015).

Production of NO contributes to immune suppression by affecting IL-2R signaling (Mazzoni et al., 2002) and by inducing apoptosis of CD8<sup>+</sup> T cells (Michaeli et al., 2017). TAN contribute to recruitment of Treg into tumors via secretion of CCL17, (Mishalian et al., 2014). They also activate Treg via CD40-CD40L interactions (Pan et al., 2010). And lastly, MDSCs up-regulate PDL-1, the ligand of the inhibitory receptor PD-1 expressed by T cells as a mechanism of retro control (Lu et al., 2016; Yamauchi et al., 2018).

#### ➤ **Metastasis**

Metastasis is a multistep process that requires tumor cells to detach from the primary tumor and migrate to target organs through systemic circulation. A body of evidence demonstrated that a supportive microenvironment, so-called pre-metastatic niche, is formed in the target organs and predispose them to metastases (Joyce and Pollard et al., 2009; Psaila and Lyden et al., 2009). In response to G-CSF derived from tumor, neutrophils are mobilized from bone marrow to pre-metastatic niche and swarm at the metastatic site before tumor cells arrive (Kowanetz et al., 2010). IL-1 $\beta$  present at high concentration in the tumor microenvironment elicit  $\gamma\delta$  T cells to produce IL-17 which triggers G-CSF responsible for the mobilization of neutrophils (Coffelt et al., 2015). Neutrophils that are recruited at the metastatic niche have been shown to promote extravasation of tumor cells into tissues, this involves interactions

between  $\beta 2$  integrin on neutrophils and ICAM-1 on tumor cells, facilitating anchoring to the vascular endothelium (Huh et al., 2010; Spicer et al., 2012). They also limit anti-tumoral CD8<sup>+</sup> T cell responses in an iNOS-dependent mechanism (Coffelt et al., 2015). In the circulation, NETs derived from neutrophils are capable of trapping tumor cells and sequester them at distant sites, thus promoting metastasis (Cools-Lartigue et al., 2013). More recently, strong evidence for a role of neutrophil in the initiation of metastasis were provided and it was found that it is mediated by leukotriene-generating enzyme arachidonate 5-lipoxygenase (Alox5) (Wculek and Malanchi et al., 2015).

### **3.5. Neutrophil polarization and neutrophil subsets in cancer**

Neutrophils exert multiple functions and a dual role in tumor biology. It remains unclear whether anti-tumoral and pro-tumoral functions are played by specific subsets or whether they display high level of plasticity and can switch from one phenotype to the other under the influence of tumor-specific factors. Linked to this issue is the question of the identity of PMN-MDSC and protumoral TAN N2 that is still not answered. Many studies have attempted to evaluate neutrophils according to different parameters such as cell surface markers, cell density, cell maturity, cell residency, cell function and also according to physiological conditions like hypoxia, glucose availability, pH. It appears that all these factors contribute to polarization.

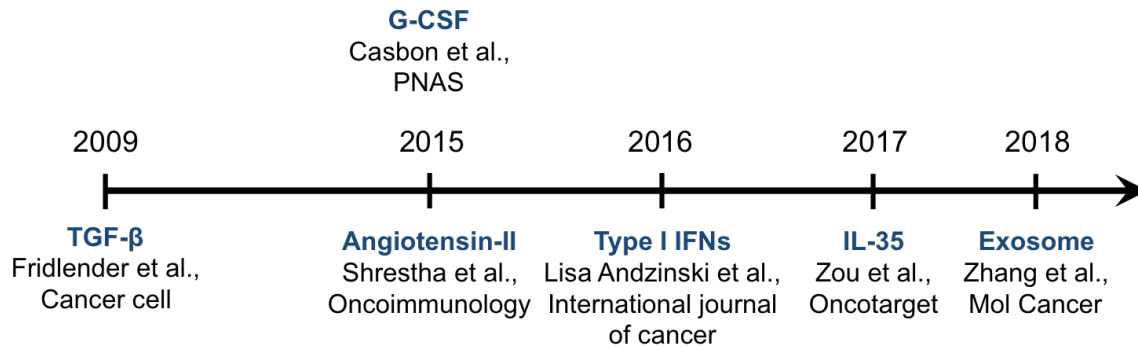
#### **3.5.1. Polarization into anti-tumoral and pro-tumoral TAN**

The first report on different neutrophil subpopulations was published in 2004 by Tsuda, Y and colleagues (Tsuda et al., 2004). They identified two new distinct neutrophils, named neutrophils I (PMN-I) present in MRSA (Methicillin-resistant *Staphylococcus aureus*)-resistant mice and neutrophils II (PMN-II) present in MRSA-susceptible mice, in addition to normal neutrophils (PMN-N) that come from naïve mice.

In cancer, the concept of polarization into N1 and N2 arose from Fridlender et al (Fridlender et al., 2009) by analogy to the polarization of macrophages into M1 and M2 (Mills et al., 2000).

This polarization is regulated by molecules present in the TME, some being listed below (Figure 11) (Piccard et al., 2012) .

**Figure 11.** Neutrophil polarization inducer



➤ **TGF-β**

Fridlender et al first demonstrated a major role for TGF-β in the polarization of neutrophils into tumor-promoting TAN (N2) (Fridlender et al., 2009). In several tumor mouse models, they administered an inhibitor of TGF-β receptor signaling which led to the recruitment of high numbers of activated cytotoxic TAN. Depletion of these TAN in treated animals using anti-Ly6G depletion led to an increase of tumor growth. Fridlender proposed to name N1, the TAN displaying anti-tumoral activities that expanded in the absence of TGF-β. In the presence of TGF-β, TAN display a pro-tumoral phenotype and were called N2. TGF-β has since been shown to control immunosuppressive functions of TAN (Coffelt et al., 2016).

➤ **Type I IFN (IFN-β)**

IFN-β has first been linked to N1 TAN when Jablonska et al demonstrated that the angiogenic activity was inhibited by IFN-β (Jablonska et al., 2010). IFN-β inhibited the accumulation of TAN at the tumor site and VEGF production and CXCR4 expression on neutrophils. IFN-β is also controlling metastasis (Wu et al., 2015) and controlling the apoptosis of protumoral TAN as *Ifnb1*<sup>-/-</sup> TANs display a prolonged lifespan (Andzinski et al., 2015). The induction of N1 polarization by IFN-β was further demonstrated both in mice and human when administration of IFN-β decreased tumor growth and reprogram anti-tumoral TAN (Andzinski et al., 2016) .

### ➤ **Angiotensin II**

Recently, Angiotensin II, a main peptide hormone of renin-angiotensin system (RAS), was shown to be responsible for the amplification of macrophage progenitor cells in the spleen and for the polarization of monocytes toward a tumor-promoting phenotype (Cortez-Retamozo et al., 2013). Based on this evidence, Shrestha et al questioned the role of Angiotensin II in the polarization of neutrophils (Shrestha et al., 2015). In the study, they used captopril, an inhibitor of Angiotensin II, that induced hyper segmentation in human neutrophils and enhanced cytotoxicity against tumor cells in a dose-dependent manner. Oral administration of captopril in a mouse 4T1 graft model attenuated tumor growth along with a significant decrease in TGF- $\beta$ 1, IL-2, IL-4, IFN- $\gamma$  in the serum, compared to control mice. The treatment also increased the percentage of splenic and intratumoral Ly6G<sup>+</sup> cells with higher nuclei hyper segmentation that produced lower level of ROS, higher level of NETs in response to PMA, and enhanced cytotoxicity. Thus, inhibition of angiotensin signaling can repolarize TAN into N1 (Shrestha et al., 2015).

### ➤ **G-CSF/ IL-35**

G-CSF is also implicated in TAN polarization. Casbon et al demonstrated that G-CSF control neutrophil differentiation towards immunosuppressive cells (Casbon et al., 2015). It has also been shown that excessive G-CSF production triggers the release of immature MDSC in the circulation that promote tumor growth (Waight et al., 2011). Recently, it was reported that IL-35, when overexpressed in tumor graft, is able to increase neutrophil infiltration and polarization to N2 TAN by increasing the production of G-CSF and IL-6. This results in the up regulation of MMP-9 and Bv8 that promote angiogenesis. Also, the down regulation of TRAIL and enhanced activation of STAT3 and ERK pathways in neutrophils, which increases the expression of iNOS suppresses T cell activation (Zou et al., 2017).

### ➤ Exosomes

Exosomes are extracellular vesicles that are present in the microenvironment and are known to have a role in cell-cell communication in tumor (Maia et al., 2018). Zhang et al observed that tumor-derived exosomes inhibited the spontaneous apoptosis and induced a pro-tumoral phenotype in neutrophils by increasing production of pro-inflammatory molecules (IL1- $\beta$ , IL-6, IL-8, TNF- $\alpha$ ), MMP-9 and VEGF and by decreasing ROS production. The tumor-derived exosomes carried the high mobility group box-1 (HMGB1) associated with immunogenic cell death and induced autophagy in neutrophils (Zhang et al., 2018).

### 3.5.2. Characterization of neutrophil subsets

In an attempt to characterize the nature of neutrophil populations described to play a role in cancer and to identify specific cell surface markers that could be associated with a specific cell subset, some research teams have undertaken gene expression profiling. A series of transcriptomic analysis have been performed in mouse tumor models and one in human. Gene signatures have been identified for specific neutrophil populations. Subsequently, some recent studies have been performed to demonstrate the specific expression of a selected marker that could discriminate N1 from N2 TAN/PMN-MDSC.

### ➤ Microarray analyses

CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>low</sup> neutrophils isolated from spleens of EL-4 tumor-bearing mice (named G-MDSC) and from casein-induced inflamed peritoneum (named Neu) were first analyzed by microarray analysis (Youn et al., 2012). A high number of differentially expressed genes (DEGs) was found and the cells diverged in phenotype and functions. G-MDSC was associated with a certain degree of immaturity, Neu with an activation state. At the same time, a second study analyzed bone marrow neutrophils from naïve mice (named NN) and spleen neutrophils (named G-MDSC) or tumor neutrophils (named TAN) from AB12 mesothelioma-bearing mice (Fridlender et al., 2012). Interestingly, TAN were the most divergent population, G-MDSC and NN being more closely related. This result may relate to the isolation of NN from the bone

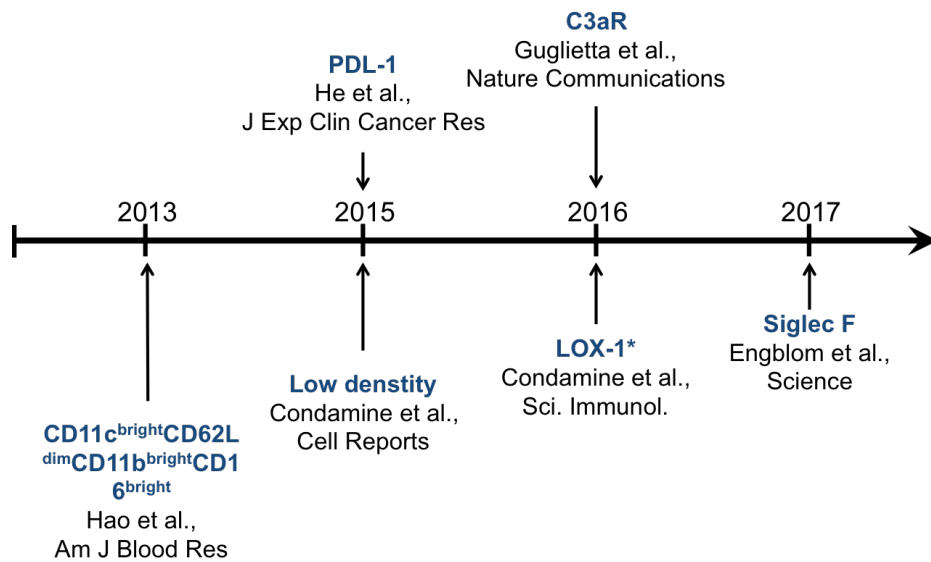
marrow and G-MDSC were previously described as immature myeloid cells. By contrast, TAN exhibited increased expression of genes associated with immune responses and decreased expression of genes associated with cell cytotoxicity. Later in 2014, an extended study analyzed multiple myeloid subsets in murine 4T1 breast cancer, Her2<sup>+</sup> breast cancer and B16F10 melanoma models (Elpek et al., 2014). The frequencies of TAN, TAM and DC differ in each tumor model and their analysis suggested that tumor type rather than anatomic location shapes the immune infiltrate. TAN displayed a specific protumoral phenotype with unique expression of haptoglobin in TAN from EL-4 tumors. Recently, Shaul et al purified N1 and N2 TAN from AB12 mesothelioma-bearing mice (Shaul et al., 2016). To do so, they treated a group of mice with an inhibitor of TGF- $\beta$  receptor that was shown to generate N1 TAN within tumors (Fridlender et al., 2009). N2 TAN were purified from untreated mice. Microarray analyses revealed a distinct gene signature for N1 and N2 TAN that was also different from bone marrow neutrophils isolated from naïve mice. N1 displayed up-regulation of genes associated with phagocytosis and inflammatory responses while N2 had down-regulation of genes associated with immune responses and showed protumoral phenotype. One study performed a microarray analysis on human high density PMN that were isolated from blood of healthy donors, high density PMN and low density PMN-MDSC isolated from blood from head and neck cancer (HNC) and non-small cell lung cancer (NSCLC) patients (Condamine et al., 2016). Interestingly, PMN-MDSC displayed the most divergent gene expression profile while PMN from healthy donors and cancer patients were closely related. The most up-regulated genes were from the ER stress response and immune response.

These studies have provided insights into the biology of TAN and mostly revealed a high plasticity of neutrophils and the need to compare populations originating from the same tissue or the same tumor model.

➤ **Specific markers that discriminate neutrophil subsets**

Many studies were conducted to identify specific markers discriminating anti- and pro-tumoral neutrophils and PMN-MDSC. Several subsets and some markers have been identified and are summarized in Figure 12.

**Figure 12.** Pro-tumor neutrophil markers



**CD11c<sup>bright</sup> CD62L<sup>dim</sup> CD11b<sup>bright</sup> CD16<sup>bright</sup> neutrophils**

In 2013, Hao et al detected a subset of neutrophils, defined to be mature and to express CD11c<sup>bright</sup> CD62L<sup>dim</sup> CD11b<sup>bright</sup> CD16<sup>bright</sup> in peripheral blood samples from 31 solid cancer patients. This subset was absent in the blood of healthy donor (Hao et al., 2013). Interestingly, this subset of neutrophils was already identified in circulating blood samples from healthy donor after systemic lipopolysaccharide (LPS) administration. In vitro, they exhibited an immune suppressive function by inhibiting T cell activation and proliferation induced by phytohaemagglutinin (PHA) or CD3/CD28 cross-linking in a Mac-1 dependent manner (Pillay et al., 2012).

### **Low-density neutrophils**

Sagiv et al observed that when isolating neutrophils from whole blood of tumor-bearing mice using density gradient, a fraction of neutrophils was found to co-purify with low-density mononuclear cell layer (Sagiv et al., 2015). A similar population was described in the blood of human cancer patients. In this study, they demonstrated that this low density CD11b<sup>+</sup> Ly6G<sup>+</sup> neutrophils (LDN) contained two cell subsets: PMN-MDSC and pro-tumoral mature neutrophils, differentiated according to their nuclei segmentation (Sagiv et al., 2015). However, they did not have any marker to distinguish them and the whole LDN population showed pro-tumor functions such as decreased phagocytosis, cytotoxicity, ROS production, and increased T cell proliferation. HDN displayed increased cytotoxicity.

### **Programmed death-ligand 1 (PD-L1)**

Overexpression of PD-L1 contributes to evasion of the immune system in cancer (Freeman et al., 2000; Gao et al., 2009). He et al investigated the expression of PD-L1 on TAN from 149 patients with hepatocellular carcinomas (HCC) and from hepatoma-bearing mice. They detected PD-L1<sup>+</sup> TAN in the peritumoral region of those patients, which could inhibit T-cell activation and proliferation. The suppression was reversed by anti PD-L1 antibodies. They demonstrated that these N2 PD-L1<sup>+</sup> TANs could be induced from human naïve neutrophils in culture with GM-CSF or TNF- $\alpha$  (He et al., 2015). Other studies have also reported up-regulation of PD-L1 on MDSCs (Iwata et al., 2016; Lu et al., 2016; Yamauchi et al., 2018). Iwata et al found the M-CSF and VEGFA could induce PD-L1 expression in vitro (Iwata et al., 2016).

### **Lectin-type oxidized LDL receptor-1 (Lox-1)**

Using a whole genome analysis, Condamine et al compared gene expression in three subsets of neutrophils: in PMN-MDSC from HNC and NSCLC patients (low-density neutrophils copurified with PBMC after density gradient); in PMN from HNC and NSCLC patients (high-density neutrophils) and in PMN (high-density neutrophils) from healthy donor (Condamine et al., 2016). They discovered Lox-1 gene that was differentially expressed and up-regulated in PMN-MDSC compared to two other subsets of neutrophils. A fraction of neutrophils expressing Lox-1

was found in blood of patients with NSCLC (4,9% among total neutrophils), HNC (6,4% among total neutrophils) and CC (6,5% among total neutrophils). Lox-1<sup>+</sup> and Lox-1<sup>-</sup> neutrophils were functionally different. Lox-1<sup>+</sup> neutrophils displayed an immunosuppressive behavior in MLR test, produced a higher level of ROS, Arginase I and iNOS. However, they could not find the same association of Lox-1 with PMN-MDSC in mice (Condamine et al., 2016).

### **Complement C3a receptor (C3aR)**

Neutrophils are known to play a crucial role in cancer-associated thrombosis by releasing neutrophil NETs (Demers et al., 2012). Guglietta et al studied the interaction between neutrophils and coagulation in a spontaneous small intestinal tumor model (Guglietta et al., 2016). Tumor progression was linked to hypercoagulation and increase of low-density N2 neutrophils with a lower production of ROS. They found that circulating LPS induced C3aR expression on neutrophils and activation of the complement cascade. This triggers NETosis and promoted tumor growth.

### **Siglec F**

Siglec-F, a specific marker of eosinophils, was found to be expressed in a proportion of neutrophils in lung adenocarcinomas (Engblom et al., 2017). The tumor stimulated bone-resident osteocalcin-expressing osteoblastic cells in the bone marrow to trigger the production of these Siglec F<sup>+</sup> neutrophils. In the lung carcinoma, these Siglec F<sup>high</sup> TAN exhibited a gene expression profile associated with protumoral functions such as angiogenesis, myeloid cell differentiation and recruitment, extracellular matrix remodeling, suppression of T cell responses, tumor cell proliferation and growth. Functionally, they produced a higher level of ROS and accelerated tumor growth compared to their control counterpart Siglec F<sup>low</sup> neutrophils (Engblom et al., 2017).

### 3.6. Potential of neutrophils in immunotherapy

Recently, TAN have been considered as targets for immunotherapy in cancers (Gregory and Houghton, 2011). In addition to numerous evidence for their infiltration in tumors, their role in immunosuppression of the anti-tumoral immune response and their tumor-promoting functions, several meta-analysis of human cancer patient cohorts have highlighted the role of TAN and the relevance to target them. Gentle et al conducted a meta-analysis on 39 human cancers in which tumor gene expression profiles (GEPs) and overall survival data were collected from 18000 patients and analyzed by a method called CIBERSORT (Cell type Identification By Estimating Relative Subsets Of known RNA Transcripts) (Lu et al., 2016; Newman et al., 2015; Yamauchi et al., 2018) to investigate the link between disease outcome and abundance of tumor-associated leukocytes (Gentles et al., 2015). Relying on relative expression of cell type specific signatures, this approach allowed them to quantify cell abundance in bulk tumors. They found that TAN infiltration was linked to the worst prognostic. A Meta-analysis of data from 8 studies of 442 patients with solid tumors showed that MDSC were associated with a poor overall survival (Zhang et al., 2016). However, TAN are not always associated with poor prognosis (Soo et al., 2018). The finding that a majority of studies link infiltration of TAN with poorest clinical outcome is also consistent with several reports associating infiltration of TAN with later stages of the disease (Eruslanov et al., 2014b; Faget et al., 2017).

Based on all these evidence, it appears that targeting TAN could be of benefit for the patients. Caution must be taken as neutrophils are critical for host defense. **One strategy to block their recruitment to the tumor site** has been considered. IL-8 antagonists have been tested and reduce tumor growth, angiogenesis and metastasis in human melanoma (Huang et al., 2002). Reparixin, a non-competitive allosteric blocker of CXCR1 and CXCR2 signaling is currently tested in clinical trials for triple-negative and metastatic breast cancers (Bertini et al., 2004). Anti-CXCR2 also block the recruitment of neutrophils in cSCC and reduce tumor growth (Jamieson et al., 2012). Another class of CCR5 antagonists has been tested and showed efficient decrease of tumor growth and metastasis (Robinson et al., 2003; Tan et al., 2009; Velasco-Velázquez et al.,

2012). Other strategies could be to **stimulate specifically N1 TAN**. TLR stimulation has been exploited in immunotherapy. Humbert et al recently demonstrated that intra-tumoral injection of TLR9 ligand reduces tumor growth via the recruitment of highly activated neutrophils that contribute to reprogram the adaptive anti-tumoral response (Humbert et al., 2018). Also, in mouse mammary carcinoma, colon adenocarcinoma and B16-F10 melanoma models, the treatment with a combination of the inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) envelope (HVJ-E) and Poly I:C, agonist TLR3, could synergistically delay tumor growth and the effect was abrogated by neutrophil depletion (Chang et al., 2016). The author proposed a mechanism for this synergic anti-tumor effect: Poly I:C increased CXCL-2 level and recruited neutrophils into the tumor and HVI-E could stimulate at least *in vitro* anti-tumorigenic activity in naïve bone marrow neutrophils by up-regulating the expression of ICAM-1 and FAS (Chang et al., 2016). Sterile inflammation is a phenomenon in which tissue damage occurs in the absence of pathogens (Kono and Rock, 2008). In such situation, a rapid infiltration of neutrophil is constantly observed and has a crucial role in inflammation resolution. Radiation has been shown to induce a similar response. Precisely, after whole-body ionizing radiation in mice, a flux of neutrophils into the spleen, thymus, and gut is detected (Lorimore et al., 2001; Nakayama et al., 2011). Takeshima et al demonstrated that radiation therapy resulted in an influx of neutrophils that contributed to the activation of tumor-specific cytotoxic T cells and tumor regression (Takeshima et al., 2016). They noted an increase of ROS and apoptosis of tumor cells that was potentiated by the combination with G-CSF treatment. G-CSF treatment triggers granulopoiesis and release of neutrophils in blood. This has demonstrated anti-cancer effects (Souto et al., 2011).

**Targeting N2 TAN** has also been tested, either to inhibit them, or to deplete them. Inhibition of PI3 kinase has shown efficacy to reduce recruitment and functions of MDSC/N2 TAN especially when used in combination with anti-PDL1 (Davis et al., 2017). Some chemotherapies have reduced or depleted neutrophils infiltrating tumors. Inhibition of phosphodiesterase-5, class I histone or STAT3 have also proved efficacy at inhibiting the immunosuppressive functions of N2 TAN (Fleming et al., 2018).

# **RESEARCH OBJECTIVES AND THESIS OUTLINE**

## Research objectives

Tumor establishment and progression results from the interplay between tumors and a variety of signals and cell types that compose the TME. As described above, neutrophils infiltrating tumors (TAN) show a remarkable plasticity that is influenced by the type of cancer, the microenvironment and the experimental model. TAN evolve along with this complex microenvironment that progress over time.

My PhD project aims to **bring additional knowledge and better understanding of TAN in cutaneous squamous cell carcinoma (cSCC)**. This type of cancer is characterized by a high mutation burden linked to chronic skin damage from UV light. The disease risk is also highly correlated with immunosuppression which suggests that immune surveillance is crucial to maintain integrity of the skin. cSCC have been reported to be highly infiltrated with immune cells (Freeman et al., 2014). Interestingly, Seddon et al observed that risk factors of cSCC recurrence, metastasis and patient death is associated to circulating G-MDSC and neutrophils (Seddon et al., 2016). But extensive study of the immune infiltrate in cSCC is missing and we focused our work on the characterization of neutrophils and their role in cSCC progression.

The following specific questions have been addressed:

- cSCC seem to be infiltrated with neutrophils but to which extend?
- What are the gene expression profiles of neutrophils that infiltrate cSCC at the precancerous and tumor stages?
- What are the gene expression profiles of neutrophils that infiltrate the skin surrounding these lesions?
- From these transcriptomic studies, can we use bioinformatics tools to reveal specific functions of TAN at each stage of carcinogenesis?
- Are these differentially expressed genes functionally relevant and can we demonstrate specific functions of TAN that contribute to tumor progression?
- In cSCC, which roles play TAN in the regulation of tumor growth and the modulation of adaptive anti-tumor immunity?

- Can we identify novel therapeutic strategy targeting neutrophils?

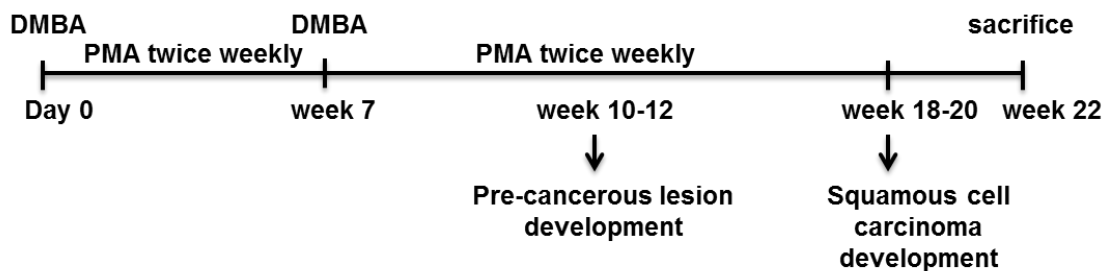
To answer these questions, I have performed the research work which is summarized in the included article currently under preparation for submission to International Journal of Cancer. The experimental models and outline of the work are summarized below.

### Experimental models

As experimental approach, we chose two mouse models:

- The chemically-induced cSCC using DMBA/PMA application on the skin of FVB/N mice (Abel et al., 2009; Owens et al., 1999)
- The intradermal transplantation of the mSCC38 cell line that was established from DMBA/PMA-treated FBV/N mice (Gastaldi et al., 2014).

➤ DMBA/PMA induced carcinogenesis is a mouse skin model of multi-stage chemical carcinogenesis which is well-established and recapitulate the human pathology (Abel et al., 2009; Owens et al., 1999). In the protocol, tumor development is initiated by two repetitive topical applications of a sub-carcinogenic dose of a carcinogen, 7,12-dimethylbenz(α)anthracene (DMBA) on mouse skin. Tumor promotion is elicited by repetitive application of a promoting agent, the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (PMA).



During the initiation stage, DMBA induces mutations of the key oncogene, Hras 1, in epidermal keratinocytes (a transversion A to T in codon 61). Additional mutations have also been reported in Kras. Between 10-12 weeks of PMA application that promote inflammation, papillomas begin to arise and a fraction of them further develop into cSCC after 18-20 weeks. Mice are generally sacrificed after 22 weeks.

➤ A murine cSCC cell line named mSCC38 was established from tumors generated by the DMBA/PMA protocol in FVB/N mice (Gastaldi et al., 2014). cSCC tumors from DMBA/PMA-treated mice were excised, and cells were put in culture. A tumorigenic cell line was cloned after successive passages. mSCC38 exhibit mesenchymal-like phenotype. They express vimentin but not E cadherin. They express constitutively an activated form of Hras (codon 61 mutated) and they are tumorigenic in mice. We implanted this mSCC38 cell line intradermally in the skin of FVB/N mice and monitored tumor growth during 28-30 days.

### **Outline of research work**

- **Axis I:** the aim was to analyze the transcriptome obtained from flow cytometry-sorted neutrophils purified from papillomas (precancerous stage), tumors (cancerous stage) and their respective surrounding skin.
- **Axis II:** the aim was to study the role and function of TAN in mSCC38 tumors. We firstly performed *in vivo* depletion of neutrophils during the course of tumor progression to assess whether they exerted pro- or anti-tumoral functions. Secondly, we dissected the mechanisms of action of neutrophils on tumor progression, either directly on tumors or indirectly on the adaptive immune responses.
- **Axis III:** the aim was to characterize pro-tumoral markers expressed by TAN infiltrating mSCC38 tumors.



# RESULTS

## RESEARCH ARTICLE

### CATEGORY: TUMOR IMMUNOLOGY AND MICROENVIRONMENT

# Tumor-associated neutrophils actively immunosuppress T cell responses and promote cutaneous squamous cell carcinoma development

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#### Short title: PD-L1 upregulation on TAN contributes to cSCC progression

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## **What's new**

The roles of neutrophils in the tumor microenvironment still need to be explored. In this study, the authors found a massive infiltration of neutrophils within cutaneous squamous cell carcinomas (cSCC) that displays a pro-tumoral gene expression signature compared to neutrophils in the surrounding skin. They show these neutrophils favor tumor growth and inhibit anti-tumoral CD8<sup>+</sup> T cell responses, notably by upregulating PD-L1. Targeting neutrophils and immune-checkpoints are thus relevant for the treatment of cSCC.

## **Abstract**

Cutaneous squamous cell carcinoma (cSCC) development has been linked to T cell function defects but the mechanisms are not fully defined. Tumor-associated neutrophils (TAN) can exert both pro- and anti-tumoral functions. In this study, we assessed the role of TAN in cSCC progression. We report a progressive increase of neutrophils in the precancerous and established cSCC lesions from chemically-induced skin carcinogenesis. Comparative in-depth transcriptomic analyses identify a pro-tumoral gene signature of TAN in lesions compared to their respective surrounding skins, indicative of a strong pressure exerted by the local tumor microenvironment. While angiogenesis and extracellular matrix remodeling are predominant functions of the precancerous TAN, immune suppression and glycolysis signals are initiated at the precancerous stage and strongly enhanced at the tumor stage. Importantly, TAN depletion delays tumor growth and significantly increases the frequency of proliferating IFN- $\gamma$ -producing CD8<sup>+</sup> T cells, demonstrating the active role of TAN in immunosuppression. Mechanisms involve high arginase 1 activity, production of ROS and upregulation of PD-L1 on TAN, concomitantly to an induction of PD1 on CD8<sup>+</sup> T cells. Frequency of PD-L1 expressing TAN positively correlates with tumor size. These data highlight the relevance to target neutrophils and PD-L1/PD1 immune-checkpoints interaction in the treatment of cSCC.

## Introduction

The molecular basis for carcinogenesis relies on multiple mutations that induce transformation of normal host cells into invasive and metastatic cancer cells. While these subsequent mutations drive progressively to carcinogenesis, the local immune system gets activated and various cellular actors are recruited. Infiltrating immune cells can either inhibit or promote tumor cell growth, notably through their participation to chronic inflammation. Non-melanoma skin cancers including basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (cSCC) result from chronic exposure to ultraviolet radiation and alterations in immunity that favor inflammation and cSCC development. A link with defective T cell responses has been demonstrated in immunosuppressed organ transplant patients that display high incidence of cSCC (Berg and Otley, 2002; Euvrard et al., 2003). Restriction of anti-tumor responses may arise from regulatory T cells that have been shown to be highly recruited within cSCC (Clark et al., 2008). However, other mechanisms still need to be explored, in particular the role of neutrophils that make up a significant proportion of immune cells infiltrating tumors. Both pro- (N2) and anti-tumoral (N1) functions have been assigned to tumor-associated neutrophils (TAN) (Fridlender et al., 2009), similarly to M1 and M2 tumor-associated macrophages (Mills et al., 2000). Their polarization seems to be regulated by the microenvironment and in particular by the presence of TGF- $\beta$  favoring N2 TAN. This functional plasticity participates to the current controversy on the role of neutrophils in cancer. The anti-tumoral function of TAN is supported by *in vivo* depletion experiments leading to failure to control tumor growth in several tumor models (Ackermann et al., 1989; Liu et al., 2016). This was linked to their capacity to kill tumor cells and inhibit their growth. The pro-tumoral role of TAN has also been documented in many tumor models. TAN promote angiogenesis, in particular via the secretion of MMP9 and VEGF (Bekes et al., 2011; Jablonska et al., 2010; Nozawa et al., 2006). TAN favor tumorigenesis, cancer cell invasion and metastasis, and stimulate tumor growth through the secretion of extracellular matrix remodeling enzymes and proangiogenic factors (Brandau et al., 2013; Piccard et al., 2012). TAN also participate to immunosuppression within tumors as a population of myeloid-suppressive cells was found

to be of granulocytic origin (Gabrilovich and Nagaraj, 2009; Youn et al., 2012). More recently, a meta-analysis of gene expression signatures among 39 human malignancies has revealed that within the leukocyte population in the tumor microenvironment, polymorphonuclear (PMN) cells have been linked to the most adverse prognostic (Gentles et al., 2015). This finding underlines the dominant role of pro-tumoral neutrophils in human cancer. In cSCC, few studies have addressed the role of TAN and blockade of their recruitment with an anti-CXCR2 antibody decreased cSCC progression suggesting TAN promote tumor growth (Jamieson et al., 2012).

We questioned the function of TAN in mouse models of cutaneous squamous cell carcinomas (cSCC), either chemically-induced with DMBA/PMA as it mimics the different stages of skin carcinoma development in humans (Abel et al., 2009; Owens et al., 1999), or using an intradermic engraftment of DMBA/PMA-derived SCC cell line (Gastaldi et al., 2014). Previous transcriptomic analyses that studied neutrophils/G-MDSC and TAN isolated from different locations such as blood, bone marrow (BM), spleen and tumor, identified a high diversity of phenotype and functions (Fridlender et al., 2012; Lakschevitz et al., 2015; Youn et al., 2012). To avoid this caveat, we performed a transcriptomic analysis on TAN populations that were isolated from the same tissue location, the skin, at two stages of tumor progression (precancerous lesions and established tumors), and either inside lesions or in the skin surrounding lesions. Interestingly, we show that the majority of TAN within precancerous lesions and tumors display a pro-tumoral phenotype that differs significantly from neutrophils isolated from their surrounding skin. We also define key features of pro-tumoral TAN in cSCC that highlight the functional relevance to target neutrophils in this cancer.

## **Materials and methods**

### **Mice**

FVB/N wild-type (WT) mice (Charles River Laboratories, L'Arbresle, France) were bred and housed in specific-pathogen-free conditions. Experiments were performed using 6-7 week-old female FVB/N, in compliance with institutional guidelines and have been approved by the regional committee for animal experimentation (reference MESR 2016112515599520; CIEPAL, Nice Côte d'Azur, France).

### ***In vivo* tumor growth**

Multi-stage chemical carcinogenesis was induced according to (Abel et al., 2009; Owens et al., 1999). 200 nmol of DMBA (Sigma-Aldrich) dissolved in toluene (Merck Millipore) and further diluted in acetone was applied on the shaved-back of mice at 6 and 13 weeks of age, together with the application twice weekly of 5 nmol of PMA (Sigma-Aldrich) dissolved in acetone, from week 1 to week 18-20. Mice were assessed for papilloma and tumor development throughout the treatment. The lesions were counted and analyzed from week 10-12 up to sacrifice week 22. mSCC38 tumor cell line was established from DMBA/PMA induced sSCCs (Gastaldi et al., 2014) and maintained in DMEM (Gibco, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GE Healthcare, Chalfont St Giles, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco).  $5 \times 10^5$  mSCC38 were intra-dermally injected in anesthetized mice after dorsal skin shaving. Tumor volume was measured manually using a ruler and calculated according to the ellipsoid formula  $\text{Volume} = \text{Length (mm)} \times \text{Width (mm)} \times \text{Height (mm)} \times (\pi/6)$ .

### ***In vivo* neutrophil depletion**

Mice received intraperitoneal injection of 150 µg of either anti-Ly6G (clone 1A8) or isotype control (clone 2A3) (BioXcell) one day before mSCC38 engraftment and continuously every three days. Neutrophil depletion was monitored by flow cytometry, in blood day 14 and 28, and in tumor at sacrifice.

### **Tissue preparation and cell purification**

Papillomas and tumors were enzymatically treated twice with collagenase IV (0.6mg/ml) (Sigma-Aldrich), dispase II (2.5mg/ml) (Roche Diagnostic) and DNase I (0.2mg/ml) (Roche Diagnostic) for 20 minutes at 37°C and skin surrounding cancerous lesions were treated twice with collagenase IV (0.6mg/ml) (Sigma-Aldrich) for 20 minutes at 37°C. Immune cells were enriched using a Percoll gradient centrifugation (GE Healthcare). For DMBA/PMA-treated mice, isolated cells were incubated with 5 µg/ml anti-CD16/CD32 (2.4G2) to block Fc receptors prior to incubation with the following fluorescently-labeled antibodies to CD3 (145-2C11), CD11c (HL3), CD45 (30-F11), CD45R/B220 (RA3-6B2), CD335/NKp46 (29A1.4), Gr-1 (RB6-8C5), Ly6C (AL-21), Ly6G (1A8) and MHCII (I-A/I-E) (2G9). For mSCC-38-bearing mice, isolated cells were enriched in CD45<sup>+</sup> cells using CD45-biotin antibody (BD Biosciences) and anti-biotin magnetic beads (Miltenyi Biotec) according to manufacturer's instructions. Cells were then incubated with 5 µg/ml anti-CD16/CD32 (2.4G2) and stained with anti-CD11b (M1/70); anti-Ly6G (clone 1A8) antibodies and live/dead marker (7-AAD). All antibodies and viability markers were purchased from BD Biosciences. Neutrophils were sorted on a BD FACSAria II™ (BD Biosciences). Purity after cell sorting was > 98%.

Spleens and bone-marrow (BM) cells flushed from mice femurs were homogenized into single-cell suspensions through a 70-µm nylon mesh. Blood was collected from tail vein on heparin tubes. Red blood cells were lysed in ACK lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1mM Na<sub>2</sub> EDTA, pH 7.2). Neutrophils were enriched from BM cell suspensions using Histopaque-based density gradient centrifugation (Sigma Aldrich), as previously described (Swamydas and Lionakis, 2013).

### **Flow cytometry**

Cell suspensions were incubated with anti-CD16/32 (2.4G2) to block Fc receptors. For flow cytometry surface labelling, cells were stained with antibodies against CD3 (145-2C11), CD4 (GK1.5 and RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD25 (PC61), CD45 (30-F11), MHCII (M5/114.15.2), Gr1 (RB6-8C5), Ly6G (1A8), Ly6C (AL-21), NKp46 (29A1.4), Siglec F (E50-2440), CD274 (MIH5), CD62L (MEL-14), CD54 (3E2), CD80

(16-10A1), CD279 (J43) (BD Biosciences, Biolegend, eBioscience) and live/dead markers: 7-AAD (BD Biosciences), Zombie Aqua and Zombie NIR (Biolegend). For intra-cellular staining, cells were either stained after isolation or restimulated for 4 hr at 37°C in complete DMEM medium supplemented with 100ng/ml PMA (Sigma-Aldrich), 1µg/ml ionomycin in the presence of GolgiStop and GolgiPlug (BD Biosciences) or with 1µg/ml LPS (Invitrogen) and 3 µg/ml brefeldin A (Sigma-Aldrich). Cells were fixed and permeabilized with either BD Cytotfix/Cytoperm (BD Biosciences) for cytokine staining or eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience) for nuclear staining. Antibodies against Foxp3 (MF23), Ki67 (SolA15), IFN-γ (XMG1.2) and Granzyme B (NGZB) were used. Samples were analyzed on a BD LSR Fortessa (BD Biosciences) and analyzed with DIVA V8 (BD Biosciences) and FlowJo V10 software (Treestar).

### **Immunofluorescence**

mSCC38 tumors were fixed in Antigenfix (Diapath) for 1hr at 4°C, washed, and incubated in 30% sucrose (Sigma-Aldrich) overnight at 4°C. Fixed tumors were washed, embedded in OCT (Tissue-Tek) and frozen prior to cryostat sectioning. 7µm-thick cryostat tumor sections were blocked with 10% normal donkey serum in PBS, 2% BSA (Sigma-Aldrich), 1% FBS, 0,5% saponin (Sigma-Aldrich) at room temperature (RT) for 1hr. Sections were stained overnight with primary antibody (purified anti-CD8 (53-6.7) or purified anti-CD31 (390) (BD Biosciences), followed by incubation for 2hr RT with donkey anti-rat IgG-A594 (Invitrogen). After extensive washes, sections were labeled with anti-Ly6G-FITC (1A8) for 1hr RT. Nuclei staining was performed using Hoechst 33342 (Life Technologies) 5 min RT and sections were mounted with Prolong Diamond (Thermo Fisher Scientific). Slides were imaged with LSM780 confocal microscope (Zeiss) and analyzed with Fiji software.

### **Oxidative burst assay**

After surface staining, 500 000 cells were incubated for 15 min at 37°C in PSE buffer (PBS, 3% FBS, 5mM EDTA) (Sigma Aldrich) supplemented with 1µM Dihydrorhodamine (DHR) 123 and 50 µg/ml catalase (Sigma-Aldrich). Cells were subsequently washed with PSE and

the fluorescence intensity of DHR123 was read on a SP6800 Spectral Cell Analyzer (Sony Biotechnology) and analyzed with FlowJo V10 software (Treestar).

### **NADPH/NADP**

After surface staining, 500 000 cells were incubated for 30 min at 37°C in PBS supplemented with JZL1707 NAD(P)H sensor according to manufacturer's instructions (AAT Bioquest). Cells were subsequently washed with PBS and the fluorescence intensity of JZL1707 NAD(P)H sensor was read in PE channel on a SP6800 Spectral Cell Analyzer (Sony Biotechnology) and analyzed with FlowJo V10 software (Treestar).

### **NO (Nitrite) quantification**

10<sup>6</sup> sorted neutrophils were cultured for 24hr at 37°C in DMEM without red phenol (Gibco) supplemented with 10% FBS (GE Healthcare), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco). NO production was measured in the supernatant using Greiss reagent assay according to manufacturer's instructions (Sigma Aldrich). The absorbance was measured at 550 nm using a Multiskan FC plate reader (ThermoFisher Scientific).

### **Arginase activity assay**

Whole cell lysates of sorted neutrophils were prepared as previously described (Germain et al., 2010). The arginase activity level was determined using Arginase Assay Kit according to manufacturer's instructions (Sigma Aldrich). The absorbance was measured at 450 nm using a Multiskan FC plate reader (ThermoFisher Scientific).

### **TGF-β1 ELISA**

Harvested tumors were immediately frozen in nitrogen liquid and thawed when cell lysates were prepared. A mechanical cell disruption and cell lysis was performed on a FastPrep-24 cell disruptor (MP Biomedical). Tumors were homogenized in 1% Igepal CA-630 (Sigma-Aldrich), 10 mM Tris-HCl pH 8, 150mM NaCl, 5mM EDTA, 10% Glycerol, 0.5% anti-mousse (silicone), protease inhibitor mixture (Complete Mini tablet, Roche Applied Science) and mixed with 0.5g Lysing Matrix D beads (MP Biomedicals) before mechanical disruption.

Supernatants were collected after centrifugation. Serum was obtained from whole blood using collecting tubes (Sarstedt). Total protein quantification was performed using BCA protein assay (Thermo Fisher Scientific) and TGF- $\beta$ 1 concentrations determined by ELISA (Invitrogen), according to manufacturer's instructions.

### **RNA isolation and microarray analysis**

50 to 700 ng total RNA was isolated from 0.01 to 2 million sorted cells using microRNAeasy kit according to manufacturer's instructions (Qiagen, Courtaboeuf, France). Purity and quality were assessed with a Bioanalyser (Agilent, Massy, France). Probes were synthesized from RNA with the LowInput QuickAmp Labeling Kit (Agilent Technologies). cRNA with appropriate quality control were hybridized to SurePrint G3 Mouse GE 8x60K microarrays (Agilent Technologies). Two or three biological replicates were performed for each experimental condition to obtain sufficient material. Neutrophils were purified from skin surrounding papillomas (3 mice per experiment), skin surrounding tumors (a mean of 5 mice per experiment), papillomas (a mean of 60 papillomas from 3 mice) and tumors (a mean of 12 tumors from 5 mice). The obtained microarray experimental data and associated microarray designs are deposited in the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under the serial record number (*to be deposited*). The raw data were quantile normalized using the Bioconductor package limma (Diboun et al., 2006). Batch effect induced by the microarray chips was removed using the ComBat method (Johnson et al., 2007). Gene expression was implemented with the limma package (Ritchie et al., 2015). The Benjamini-Hochberg procedure was used to control the experiment-wise false discovery rate (FDR) from multiple testing procedures. We performed a principal component analysis (PCA) on the 15% most variable probe sets with a minimum log<sub>2</sub> average expression of 6, selected with the PopulationDistances program from <http://cbdm.hms.harvard.edu/LabMembersPges/SD.html>. Using a pairwise average-linkage method (GenePattern) (<http://www.broadinstitute.org/cancer/software/genepattern/>) (Reich et al., 2006), a hierarchical clustering was performed on the same probe sets median-centered across all samples. Differentially expressed probe sets were selected based on an average log<sub>2</sub>

expression level across all conditions of at least 6, an absolute log<sub>2</sub>-fold change  $\geq 1$ , and an adjusted *P* value  $\leq 0.05$ . Further analyses were carried out using Protein Analysis Through Evolutionary (PANTHER) database (<http://pantherdb.org>) (Mi et al., 2016) and QIAGEN's Ingenuity Pathway Analysis (IPA) tool, QIAGEN Redwood City, (<http://www.qiagen.com/ingenuity>). A biological correlation coefficient between papilloma versus papilloma skin and tumor versus tumor skin comparisons was estimated using the Genuine Association of Gene Expression Profiles (GENAS) method implemented in the limma R package (Majewski et al., 2010). A k-means ++ (z-norm) clustering was performed with the ExpressCluster software v1.3 (<http://cbdm.hms.harvard.edu/LabMembersPges/SD.html>) on the union of differentially modulated genes between lesions and surrounding controls (a total of 2,166 probe sets). For genes with multiple probe sets, the median intensity value was computed.

### **Statistical analysis**

Statistical analyses were carried out with Prism software version 6.0 (GraphPad Prism). Depending on data distribution (Shapiro normality test) and matched or not matched observations, a Kruskal-Wallis test, an unpaired t test, or a Mann Whitney test, were used. Pairwise multiple comparisons of experimental groups were performed using Two-way ANOVA. *P*  $\leq 0.05$  was considered statistically significant.

## Results

### **Profiling of tumor-associated neutrophils (TAN) in the cutaneous squamous cell carcinoma (cSCC) lesions**

Application of DMBA together with PMA on mouse skin is a widely used experimental model to study skin tumorigenesis. This model accurately mimics the different stages of skin carcinoma development in humans, by inducing papillomas which can further develop into cSCC (Supporting information Fig. S1A). To assess the role of TAN in cSCC, we focused our study on Gr-1<sup>bright</sup> TAN that extravagated into tissue, either within the skin surrounding precancerous lesions (papilloma skin) and established carcinomas (tumor skin), or those infiltrated in the precancerous lesions (papilloma) and in the carcinomas (tumor). We detected a significant and progressive increase of the proportion of TAN within papillomas and tumors compared to surrounding skin, with TAN accounting for up to ~50% of tumor-infiltrating CD45<sup>+</sup> cells (Fig. 1a). These Gr-1<sup>bright</sup> cells exclusively expressed Ly6G confirming they were neutrophils and not monocytes (Supporting information Fig. S1B) (Daley et al., 2008). To apprehend the role of these specific neutrophil populations within the tissue where carcinogenesis occurs and throughout the process of tumor development, we used an unbiased gene expression array analysis approach on highly purified Gr-1<sup>bright</sup> cell populations (Supporting information Fig. S1C). Global gene expression analysis by principal component (PCA) of the 15% most variable probe sets displaying a minimum log<sub>2</sub> average expression of 6 revealed a clear segregation (Fig. 1b). The first principal component axis (PC1), the one explaining the largest percentage of variability (69.05%), separated the control skin samples from cancer lesion stages. The identity of papilloma versus tumor stages was illustrated by the PC2, explaining 15.76% of variability. The unique gene-expression profile of each cell origin was further emphasized in the hierarchical clustering of previously selected 15% most variable probe sets (Supporting information Fig. S1D).

We next examined the differentially expressed genes (DEGs) in pairwise comparisons (Fig. 1c). The most divergent contrast was the comparison of tumor with its control tumor skin yielding 2045 DEGs, followed by the comparison of papilloma with control papilloma skin

yielding 1528 DEGs. In these two contrasts, the majority of DEGs were upregulated. To a lesser extent, we observed 235 and 144 differentially modulated genes for the contrasts tumor versus papilloma, and tumor skin versus papilloma skin, respectively. A majority of genes were downregulated in the contrast tumor versus papilloma. Using Genuine association of expression profiles (GENAS) method, we also compared the log fold changes between the two most divergent contrasts on the totality of probe sets, without setting differential expression cutoffs (Supporting information Fig. S1E). The GENAS analysis yielded a biological correlation coefficient of 0.76 ( $P < 0.001$ ) indicating that gene modulation in the tumor TAN followed the same trend as in the papilloma TAN.

Altogether, these analyses identify a massive infiltration of TAN in cSCC lesions with distinct gene signature for TAN infiltrating papillomas and tumors compared to their skin counterparts, thus highlighting a strong impact of the local microenvironment.

### **Gene expression patterns define pro-tumoral functions of TAN in cSCC with specific functions at the pre-cancerous and cancerous stages**

To apprehend biological processes and molecular functions associated with TAN in the context of cutaneous carcinoma pathogenesis, we performed a core analysis of the DEGs using Ingenuity Pathways analysis (IPA). As shown in Fig. 1d, TGF- $\beta$ , TNF- $\alpha$  and IFN- $\gamma$  were the most significantly activated upstream regulators in lesions compared to surrounding skin. These cytokines have been associated with pro-tumoral phenotypes (Fridlender et al., 2009; Glodde et al., 2017). Our data therefore suggest that the cSCC microenvironment is favoring the development of pro-tumoral TAN. The canonical pathways enriched and modulated in at least one of the three contrasts ( $P \leq 0.05$ , absolute z-score  $\geq 2$ ) (Supporting information Fig. S2A) also highlighted pro-tumoral functions for TAN. IL-8 signaling was the canonical pathway with the most significant  $P$  value in contrasts comparing lesions to their control skin (Supporting information Fig. S2B). The differential gene expression revealed that signaling through CXCR1 and CXCR2 IL-8-receptors expressed by TAN resulted in the activation or inhibition of specific functions promoting tumor growth (Supporting information Fig. S2B). The inhibition of apoptosis suggested that TAN had an

increased lifespan within the tumor microenvironment compared to surrounding skin. Neutrophil adhesion and chemotaxis were activated, consistent with the well-known role of IL-8 in the recruitment of neutrophils to the tumor site (De Larco et al., 2004) and in correlation with the increased number of TAN found in lesions compared to surrounding skin (Fig. 1a). Other activated functions included angiogenesis, tumor invasion, tumor growth, inflammation, endothelial cell migration and retraction. Altogether, these results highlight prominent pro-tumoral functions of TAN in lesions compared to surrounding skin.

Besides many common biological processes suggesting an overall similar modulation of genes between lesions and their respective skin control at both stages of cSCC development (Supporting information Fig. S1E), specificity at each stage of carcinogenesis could also be found. To address this question, we applied a profile clustering method on 2,166 probe sets selected as differentially expressed in at least one of the two contrasts papilloma versus papilloma skin and tumor versus tumor skin. We obtained ten distinct clusters associated with a specific pattern of gene expression revealing a clear separation between skin controls and lesions (Fig. 1e). We performed a PANTHER enrichment analysis on each of the ten clusters (Supporting information Fig. S3). With this approach, we could retrieve both shared and specific functions of TAN and link them to stages of carcinogenesis or localizations. The GO enrichments revealed that the main features of TAN once infiltrated into the lesions were related to extracellular matrix remodeling mainly within papillomas (cluster 1 and 7), repressed cytoskeleton reorganization controlling neutrophil functions (cluster 2), repressed leukocyte extravasation and migration (cluster 3), decrease of specific inflammatory and immune responses (cluster 3 and 4), response to IL-8 and TGF $\beta$  both in papillomas and tumors (cluster 6 and 7), angiogenesis and metastasis (cluster 7), active glycolysis (clusters 5, 8 and 9) and increased apoptosis and immune suppression (cluster 10).

These analyses highlight TAN's pro-tumoral functions that evolve overtime throughout tumor progression. While TAN are actively involved in angiogenesis and extracellular matrix remodeling at the precancerous stage, they display repressed immunological functions and increased glycolysis and survival at the tumor stage.

## TAN promote tumor growth in cSCC

To further assess the role played by TAN in the development of cSCC, we took advantage of a recently established cSCC cell line mSCC38, that was generated from DMBA/PMA-treated mice (Gastaldi et al., 2014). Intradermal inoculation of tumorigenic mSCC38 promoted tumor growth in all mice, over the course of ~30 days (Fig. 2a). Like for the DMBA/PMA model, an increasing number of neutrophils infiltrated the grafted mSCC38 overtime (Fig. 2b) and they were found localized within the tumor bed (Fig 2c). Their proportion among infiltrating CD45<sup>+</sup> immune cells was positively correlated with tumor volume (Fig. 2d). Simultaneously, the frequency of neutrophils detected in blood increased overtime while no apparent changes were noted in skin, spleen and BM (Supporting information Fig. S4A). Next, we used *in vivo* depletion of neutrophils to assess the role of TAN in tumor development. We set up a protocol using anti-Ly6G mAb that allowed depletion of TAN in blood and in tumors (Supporting information Fig. S4B, S4C). As shown in Figure 2e, the depletion of TAN significantly delayed mSCC38 growth as compared to isotype control mAb-treated mice (Fig. 2e). These results indicate that neutrophils promote cSCC growth. Consistent with this phenotype, we detected TGF- $\beta$  within tumors and a 2 fold-increase between days 15 and 31 (Fig. 2f).

The mechanisms used by TAN were next investigated by assessing functional characteristics of neutrophils found in tumors compared to skin, spleen and BM from tumor-bearing mice. Mature neutrophils have been reported to constitute the main population of neutrophils in these organs but BM also host a significant proportion of precursors and immature neutrophils (Evrard et al., 2018). In our transcriptomic analysis, we found that neutrophils from both lesions and skin displayed a mature phenotype, with *Cebpd* being the sole transcription factor expressed, and genes associated with secondary and tertiary granules but not primary granules being transcribed (Supporting information Fig. S5). Reactive oxygen and nitrite species are produced by neutrophils in various conditions of activation. The intracellular level of the reduced form of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) is linked to production of ROS. Using a fluorescent sensor, we measured a significant increase of

intracellular levels of NAD(P)H in neutrophils infiltrating tumors compared to skin (Fig. 3a). Spleen neutrophils behaved like TAN while BM neutrophils had NAD(P)H levels similar to skin neutrophils. Consistent with these results, we found an elevated production of ROS in TAN, lower levels in spleen and poor ROS production in skin and BM (Fig. 3b). To further elucidate the role of TAN in cSCC, we quantified nitrite produced by cell-sorted neutrophils from tumors and BM. Because of the paucity of neutrophils in the skin, we were not able to include the skin control but we added neutrophils from BM of naïve mice. As shown in Figure 3c, 3d, TAN produced significantly more nitrite and this is consistent with an increased transcription of *nos2* in neutrophils from tumors and papillomas compared to surrounding skin. ROS and NO may contribute to both tumor killing and genotoxicity promoting tumor growth. To distinguish them, we assessed the cytotoxic activity of TAN. Recently, granzyme B has been detected in anti-tumoral neutrophils (Martin et al., 2018). Upon LPS restimulation *in vitro*, we were able to detect significantly higher levels of granzyme B expression in neutrophils from BM and spleen compared to neutrophils in skin and tumor (Fig. 3e). This differential expression suggests that TAN are poorly cytotoxic both in the tumor and in the skin.

Collectively, our data demonstrate that infiltrated TAN contribute to cSCC development through the production of ROS and NO and they display reduced cytotoxicity capacity.

### **TAN suppress of anti-tumoral CD8 T cell responses**

Suppression of T cell activity is a hallmark of MDSC function (Gabrilovich and Nagaraj, 2009). However, concerns have been raised recently over the accuracy of the *in vitro* assays used to measure immunosuppression (Negorev et al., 2018). Similarly, we obtained contradictory and inconsistent results when we used coated anti-CD3/CD28 microbeads and plates to stimulate T cell proliferation in the presence of neutrophils purified from mSCC38 tumors (data not shown). We therefore evaluated the immunosuppressive activity of TAN directly *in vivo* by comparing the adaptive immune response of tumor-bearing mice that were treated either with anti-Ly6G depleting mAb or isotype control (Fig. 4a). Flow cytometry analysis of the composition of the immune infiltrate revealed no major differences in the frequencies of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells (Supporting information

Fig. S6A). By contrast, the frequencies of proliferating CD3<sup>+</sup>CD8<sup>+</sup> T cells and IFN- $\gamma$ -producing CD3<sup>+</sup>CD8<sup>+</sup> T cells were significantly increased in the absence of neutrophils (Fig. 4a, 4b). This immune suppression may result from a cross-talk between TAN and CD3<sup>+</sup>CD8<sup>+</sup> T cells, as immunofluorescence staining of mSCC38 tumor sections identified neutrophils in contact with CD8<sup>+</sup> T cells (Fig.4c). In addition, we observed a tendency of lower frequency of highly immunosuppressive Foxp3<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Treg) among CD4<sup>+</sup> T cells in neutrophil-depleted mice, in agreement with previous reports showing that TAN contributed to Treg recruitment (Mishalian et al., 2014) (Supporting information Fig. S6B). No clear differences were observed in the frequency of natural killer (NK) cells in control and neutrophil-depleted mice (Supporting information Fig. S6C). Several mechanisms can be responsible for the inhibition of anti-tumoral CD3<sup>+</sup>CD8<sup>+</sup> T cell responses. The high number of Treg infiltrating mSCC38 recapitulate the human pathology. Human cSCC are highly infiltrated with Treg exerting immunosuppressive functions (Clark et al., 2008). TAN were found to upregulate arginase 1, both at the transcriptional and protein level (Fig. 4d, 4e). Arginase activity was significantly increased in TAN compared to neutrophils isolated from BM of naïve and mSC338-bearing mice (Fig. 4e). Arginase catalyzes the hydrolysis of arginine, an amino acid essential for T cell proliferation, thus contributing to immune suppression.

### **Concomitant upregulation of PD-L1 on TAN and PD1 on CD3<sup>+</sup>CD8<sup>+</sup> T cells within the tumor contributes to immunosuppression and tumor growth**

The phenotype and functions of TAN has been extensively studied but a clear consensus is still not reached (Moses and Brandau, 2016; Veglia et al., 2018). A few cell surface markers including LOX-1, Siglec F and PD-L1 have been proposed to distinguish specific TAN subsets but further investigation is needed to validate them (Condamine et al., 2016; Engblom et al., 2017; He et al., 2015). We also wondered whether the level of activation is linked or not to anti- and pro-tumoral functions of TAN (Gregory and Houghton, 2011). To investigate these questions, we phenotyped neutrophils from tumor, skin, blood, spleen and BM, in mSCC38-bearing mice. A restricted set of activation markers and cell surface

receptors identified on MDSC were analyzed (Supporting information Fig. S7). We concatenated all the samples and performed an unsupervised t-distributed stochastic neighbor embedding (tSNE) analysis on gated CD45<sup>+</sup>Ly6G<sup>+</sup> neutrophils (Fig. 5a). This tSNE analysis revealed a greater similarity in the expression profile of clustering markers between TAN and skin neutrophils than neutrophils from spleen, blood and BM. When we analyzed each marker individually, we noticed that neutrophils from tumor and skin shared markers associated with activation including high expression of Ly6G, CD11b, CD11c, CD54, CD80 and downregulation of CD62L (Supporting information Fig. S7A, S7B). Moreover, we detected a strong increase of PD-L1<sup>+</sup> and Siglec F<sup>+</sup> TAN, (Supporting information Fig. S7A, Fig. 5b). Siglec F was also found expressed on a proportion of neutrophils in skin, blood and spleen but not in BM, while PD-L1 seemed to be more restricted to tumor TAN. To investigate whether upregulation of PD-L1 on TAN could have a functional relevance, we analyzed the expression of PD1 on NK and T cells (Fig. 5c). A striking increase of PD1-expressing CD8<sup>+</sup> and CD4<sup>+</sup> T cells frequencies was detected ( $P < 0.0001$ ). By contrast, no expression of PD1 was reported on NK and T cells from spleen and lymph node of tumor-bearing mice. Moreover, significant positive correlations were found between tumor size and the frequencies of PD-L1<sup>+</sup> TAN, either Siglec F<sup>+</sup> or<sup>-</sup>. By contrast, a negative correlation was found with PD-L1<sup>-</sup>Siglec F<sup>+</sup> TAN and no correlation with PD-L1<sup>-</sup>Siglec F<sup>-</sup> TAN (Fig. 5d). The subsets expressing PD-L1 also produced significantly more ROS than the subsets expressing Siglec F (Fig. 5e). Collectively, these results, together with the depletion experiments (Fig. 4a) and the visualization of CD8<sup>+</sup> T cells in contact with TAN in the tumor bed (Fig. 4c) corroborate a role for PD-L1-expressing TAN in immunosuppression.

## Discussion

This study provides an extensive characterization of neutrophils infiltrating the skin and lesions during cutaneous squamous cell carcinoma development. We show that TAN actively participate to the immune suppression that promotes cSCC growth. TAN inhibit anti-tumoral CD8<sup>+</sup> T cells through several mechanisms including elevated arginase activity, ROS and NO. Importantly, we identified a specific upregulation of PD-L1 on TAN and PD1 on CD8<sup>+</sup> T cells infiltrating tumors and a positive and significant correlation between PD-L1<sup>+</sup> TAN frequency and tumor size. Together, these data underline a key role for PDL1-expressing TAN in PD-L1/PD1 axis in cSCC.

Cutaneous squamous cell carcinoma is the second most common skin cancer (Lomas et al., 2012; Rogers et al., 2015). It is generally cured with surgery but can reach a stage of advanced invasive disease associated to extremely rapid local relapse for which no efficient therapy has been approved so far. Recently, neutrophils have been associated with the most adverse clinical outcome (Gentles et al., 2015). We addressed their role in cSCC as little is known about their contribution to the disease. To date, our understanding of the role of neutrophils in cancer remains unclear. Indeed, neutrophils can exert both anti- and pro-tumoral functions and they display a high diversity and plasticity (Elpek et al., 2014; Fridlender et al., 2009; Fridlender et al., 2012; Youn and Gabrilovich, 2013). Specifically, it appears that these differences mainly relate to the type of tumor and the tissue localization. We therefore hypothesized that to obtain the most relevant and complete analysis of TAN infiltrating cSCC, they should be compared to neutrophils from the skin surrounding the tumors at the time of analysis. We were comforted in this choice when we analyzed neutrophils purified from blood which carried a much higher variability (data not shown). TAN isolated from papillomas or tumors, when compared to neutrophils from their respective skin controls, provided the highest number of DEGs. Still, some diversity was also found between papillomas and tumors and specific functions could be identified at each stage of the disease. Our data therefore not only demonstrate a pivotal role of the tumor microenvironment in shaping neutrophils, but also highlight sequential roles for TAN through tumor progression. Indeed, while angiogenesis and extracellular matrix

remodeling are predominant functions of the precancerous TAN, immune suppression and glycolysis signals are initiated at the precancerous stage and strongly enhanced at the tumor stage. These results may relate to studies in human cancer patients showing anti-tumoral functions at early-stages of the disease and pro-tumoral functions at later stages (Singhal et al., 2016). We bring an additional input here, showing sequential functions of TAN promoting tumor growth.

The nature of TAN remains a subject of controversy. They share phenotype and functions with MDSC, although MDSC have been described as immature cells (Bronte et al., 2016). A recent developmental analysis of BM neutrophils identified transcriptomic profiles for each stage of maturation (Evrard et al., 2018). We therefore confronted these profiles with our data and showed that overall, the profiles of both TAN in cSCC and neutrophils from the skin display a mature phenotype. Similarly, another issue to address is the activation state. It has been questioned whether anti- and pro-tumoral TAN could have differential levels of activation (Fridlender et al., 2009; Houghton, 2010). In human studies, anti-tumoral TAN are shown to express a “classic activated phenotype” with upregulation of CD54 and downregulation of CD62L and CD16 (Eruslanov et al., 2014; Fortunati et al., 2009). Interestingly, when we phenotyped neutrophils from several anatomic locations in mSCC38-bearing mice, we found that only TAN and neutrophils from skin shared activation markers, with TAN showing slightly higher MFI for a few markers. As TAN in our model display a clear pro-tumoral phenotype, the correlation between level of activation and anti- and pro-tumoral phenotypes needs to be revisited.

A key observation in our study is that TAN in cSCC are actively involved in the immunosuppression of anti-tumor CD8<sup>+</sup> T cell responses. This was demonstrated *in vivo* to avoid the artefacts reported in *in vitro* assays (Negorev et al., 2018). We identified several mechanisms in agreement with the literature (Coffelt et al., 2016; Gabrilovich and Nagaraj, 2009). The arginase activity and NO were measured from purified TAN and the observed significant increase correlated with the transcriptomic data. TAN also produced higher level of ROS but less granzyme B, suggesting they are less cytotoxic (Martin et al., 2018). Recently, ROS have been shown to act as intracellular signaling molecules that modulate T cell functions (Franchina et al., 2018). Currently, it is difficult to assess the exact contributions of ROS. We hypothesized that they will be diverse and they will differ locally,

being directed towards either the tumors and the cells within TME. Interestingly, ROS production was associated with PD-L1 expression induced on TAN. PD-L1 has previously been shown to be expressed on TAN in hepatocellular carcinoma (He et al., 2015), colon cancer (Lu et al., 2016) and lung cancer (Yamauchi et al., 2018). By binding to PD1 on T cells, PD-L1 contributes to the immunosuppression of T cell anti-tumoral responses. In cSCC, we show that TAN upregulate PD-L1 while CD8<sup>+</sup> T cells upregulate PD1. Importantly, we demonstrate a significant and positive correlation between the frequencies of PD-L1<sup>+</sup> TAN and tumor size. Considering the high number of TAN infiltrated in cSCC, it is likely that this mechanism contributes to immunosuppression. We also evaluated the expression of Siglec F reported to be a pro-tumoral maker for TAN in lung adenocarcinomas (Engblom et al., 2017). Siglec F was induced on a proportion of TAN in cSCC, either together with PD-L1 or alone but its expression was not associated with ROS, nor correlated with tumor size. In lung adenocarcinoma, an association with ROS was described and adoptive transfer of Siglec F<sup>+</sup> but not Siglec F<sup>-</sup> TAN accelerated tumor growth (Engblom et al., 2017). This discrepancy may reflect differences between tumor types (Elpek et al., 2014).

In conclusion, this extensive *in vivo* study of TAN within cSCC underlines their diversity of functions and highlights their predominant involvement in immunosuppression of CD8<sup>+</sup> T cell responses, particularly through upregulation of PD-L1. Interestingly, a recent phase 1 study of cemiplimab targeting PD1 was conducted in advanced cSCC and induced a response in half of the patients (Migden et al., 2018). It will be important to study the potential link with PD-L1 expression on TAN. Moreover, it is relevant to combine therapeutic approaches targeting PD-L1/PD1 and TAN.

## Figure legends

**Figure 1. Distinct transcriptional signatures of neutrophils isolated from cSCC lesions and surrounding skin highlight their pro-tumoral functions.** (a) Increase of the proportion of Gr-1<sup>bright</sup> neutrophils among CD45<sup>+</sup> cells throughout tumor progression. Percentage of Gr-1<sup>bright</sup> cells among CD45<sup>+</sup> cells in skin surrounding papillomas (papilloma skin) and tumors (tumor skin), within papillomas and within tumors, n=6 mice per group, \* $p < 0.05$ , \*\* $p < 0.01$ , Mann-Whitney  $U$  test. (b) Three Dimensional Principal Component Analysis (PCA). The 15% most variable probe sets with a minimum log<sub>2</sub> average expression of 6 were analyzed within the purified Gr-1<sup>bright</sup> cell populations. (c) Differentially Expressed Genes (DEGs) in pairwise comparisons. The four contrasts were analyzed based on a minimum log<sub>2</sub> average expression of 6, an absolute log<sub>2</sub>Fc of at least 1, and an adjusted  $P$  value  $p \leq 0.05$ . (d) IPA upstream regulators enrichment. Analysis of DEGs in papilloma versus papilloma skin (outer circle), tumor versus tumor skin (middle circle) and their pairwise comparison. The most significant ( $-\text{Log}_{10} \geq 1.39$ ) upstream regulators are shown. A negative z-score (blue) denotes an inhibited pathway. A positive z-score (orange) stands for an activated pathway. (e) Hierarchical clustering of genes differentially expressed between lesions and their respective skin control identifies 10 clusters. The DEGs were selected based on an average log<sub>2</sub> expression level across all conditions of at least 7, an absolute log<sub>2</sub>-fold change  $\geq 1$  and an adjusted  $P$  value  $\leq 0.05$ , in at least one of the two contrasts, papilloma versus papilloma skin and tumor versus tumor skin.

**Figure 2. High infiltration of protumoral TAN in cSCC.** (a) Growth of mSCC38 cell line grafted intradermally in FVB/N mice (n= 10-12 mice, mean  $\pm$  SEM from three independent experiments). (b) Frequencies of TAN overtime in mSCC38-bearing mice (n=9-12, mean  $\pm$  SEM from three independent experiments). (c) Representative immunofluorescence staining of frozen sections of mSC338 tumor at day 30 post-tumor graft. (d) Correlation between the frequency of infiltrating Ly6G<sup>+</sup> neutrophils and tumor size (n=31). Linear regression curve, spearman r value and P value (95% confidence interval) are shown. (e, left panel) Experimental protocol of the depletion of neutrophils in mSCC38-bearing mice.

(e, right panel) Fold change of tumor growth between day 17 and indicated times (n= 10 mice per group, mean  $\pm$  SEM from two independent experiments), \*\* $P$ <0.01 Two-way ANOVA. (f) Representative TGF- $\beta$  concentration in mSCC38 tumors (n=3, mean  $\pm$  SD).

**Figure 3. TAN produce ROS and NO but are poorly cytotoxic.** (a) Intracellular NAD(P)H levels, (b) ROS production in neutrophils from BM, spleen, skin and mSCC38 from tumor-bearing mice (n=8-17 mice, mean  $\pm$  SEM from two or three independent experiments). (a,b, left panel) Histogram overlays showing labelling of gated neutrophils for each sample (c) Average intensity expression levels and adjusted p-values of *Nos2* transcripts from the Agilent microarray analysis. (d) Nitrite concentrations measured in supernatants from overnight incubation of flow cytometry-sorted neutrophils from indicated mice and organs (n=18 mice, mean  $\pm$  SEM from three independent experiments). (e) Intracellular staining for granzyme B in neutrophils of BM, spleen, skin and mSCC38 from tumor-bearing mice, after *ex vivo* LPS stimulation (n=5 mice, mean  $\pm$  SD). (e, left panel) Histogram overlay showing labelling of gated neutrophils for each sample. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001 Kruskal-Wallis test.

**Figure 4. TAN suppress anti-tumoral CD8<sup>+</sup> T cell responses.** (a) Frequencies of proliferating Ki67<sup>+</sup> CD3<sup>+</sup>CD8<sup>+</sup> T cells infiltrating mSCC38 tumors in isotype control or anti-Ly6G-treated mice (n=10 mice, mean  $\pm$  SEM from two independent experiments). \*\* $P$ <0.01, Mann-Whitney *U*-test. (b) Frequencies of IFN- $\gamma$ -producing CD3<sup>+</sup>CD8<sup>+</sup> T cells infiltrating mSCC38 tumors in isotype control or anti-Ly6G-treated mice (n=5 mice, mean  $\pm$  SD). \* $P$ <0.05 unpaired Student's *t* test. Representative contour-plots are shown (a,b, left panel). (c) Representative IF staining of frozen sections of mSCC38 tumor at day 30 post-tumor graft. (d) Average intensity expression levels and adjusted p-values of *Arg1* transcripts from the Agilent microarray analysis. (e) Arginase activity measured in whole cell lysates from flow cytometry-sorted neutrophils from indicated mice and organs (n=18 mice, mean  $\pm$  SEM from three independent experiments). \* $P$ <0.05, Kruskal-Wallis test.

**Figure 5. Upregulation of PD-L1 and Siglec F on TAN is concomitant with expression of PD1 on CD8<sup>+</sup> and CD4<sup>+</sup> T cells and is associated with a positive correlation between PD-L1<sup>+</sup> TAN frequencies and tumor volume.** (a) Flow cytometry t-SNE analysis was performed on live CD45<sup>+</sup>Ly6G<sup>+</sup> neutrophils after downsampling and concatenation of all tissue samples. t-SNE analysis was run on CD11b, CD11c, CD54, CD62L, CD80, Siglec F and PD-L1 markers. Neutrophils expressing PD-L1 were visualized in red in each tissue sample. (b, left panel) Representative contour-plots of Siglec F and PD-L1 expression on neutrophils from BM and mSCC38 tumors. (b, right panel) Frequencies of neutrophils expressing either PD-L1 and Siglec F alone or together or none of them, day 30 post-tumor graft. (n=5-12 mice, mean ± SEM from two independent experiments). PD-L1<sup>-</sup>Siglec F<sup>-</sup> from tumors was significantly downregulated compared to the other subsets, \*\*\*\* $P < 0.0001$  Two-way ANOVA. PD-L1<sup>+</sup>Siglec F<sup>-</sup> from tumors was significantly upregulated compared to skin and BM, \* $P < 0.05$  Two-way ANOVA. PD-L1<sup>+</sup>Siglec F<sup>+</sup> from tumors was significantly upregulated compared to skin and spleen, \* $P < 0.05$  Two-way ANOVA and to BM, \*\* $P < 0.01$  Two-way ANOVA. PD-L1<sup>-</sup>Siglec F<sup>+</sup> from tumors was significantly upregulated compared to BM, \* $P < 0.05$  Two-way ANOVA. (c) Frequencies of PD1<sup>+</sup> cells among NK cells, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells from mSCC38-bearing mice day 30 post-tumor graft. (n=10-13 mice, mean ± SEM from two independent experiments). \*\*\*\* $P < 0.0001$  Two-way ANOVA. (d) Correlation between the frequency of infiltrating PD-L1<sup>+</sup>Siglec F<sup>-</sup>, PD-L1<sup>+</sup>Siglec F<sup>+</sup>, PD-L1<sup>-</sup>Siglec F<sup>-</sup>, PD-L1<sup>-</sup>Siglec F<sup>+</sup> neutrophils and tumor size (n=12). Linear regression curve, spearman r value and P value (95% confidence interval) are shown. (e) ROS production in TAN mSCC38 from tumor-bearing mice day 30 post-tumor graft (n=17 mice, mean ± SEM from three independent experiments) \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  One-way ANOVA.

## Supplementary Figure Legends

**Supplementary Figure S1. Flow cytometry and transcriptomic analyses of cell-sorted neutrophils in a chemically-induced cSCC. (A)** Multi-stage chemical carcinogenesis. Treatment protocol of FVB/N mice: pre-cancerous lesions (papillomas) appear ~weeks 10-12, some further converting into squamous cell carcinomas (tumor) ~weeks 18-20. **(B)** Gr-1<sup>bright</sup> cells identify Ly6G<sup>+</sup> neutrophils. Representative flow cytometry dot plots of CD45<sup>+</sup> gated cells. **(C)** Multiparameter flow cytometry cell sorting of Gr-1<sup>bright</sup> cells. Representative flow cytometry dot plots of CD45<sup>+</sup> gated cells, with Gr-1<sup>bright</sup>-sorted cells visualized within rectangles in black. **(D)** Hierarchical clustering. The 15% most variable probe sets with a minimum log<sub>2</sub> average expression of 6 were analyzed within the purified Gr-1<sup>bright</sup> cell populations. Probe sets were median-centered and a clustering per rows and columns was performed with the average-linkage method. **(E)** GENAS biological correlation between tumor and papilloma modulation on all probe sets.

**Supplementary Figure S2. IPA Canonical Pathway enrichment. (A)** Analysis of DEGs in papilloma and tumor versus their respective skin controls and their pairwise comparison. The most significant (-Log<sub>10</sub> p-value ≥ 1.39) canonical pathways are shown. A negative z-score (blue) denotes an inhibited pathway. A positive z-score (orange) stands for an activated pathway. **(B)** IL-8 Signaling Canonical Pathway. Overlay of DEGs from papilloma versus papilloma skin. Red and green colors stand for genes up and down-regulated respectively. A molecular activity predictor (MAP) was superimposed. Orange and blue stand for activation and inhibition of the gene or function.

**Supplementary Figure S3. Gene cluster GO-enrichment analyses.**

**Supplementary Figure S4. Neutrophil frequencies in mSCC38-bearing mice. (A)** Frequencies of neutrophils overtime in mSCC38-bearing mice (n=5-11, mean ± SEM from three independent experiments). **(B)** Representative contour plot showing expression of Gr1 epitope (anti-Ly6G + Ly6C) and Ly6C in CD45<sup>+</sup> cells from blood of isotype control or

anti-Ly6G-treated mice day 15 post-tumor graft. **(C)** Frequencies of neutrophils monitored as in (B) in isotype control or anti-Ly6G-treated mice. (n=5 mice, mean  $\pm$  SD).

**Supplementary Figure S5. Heat map of the expression levels of transcripts from the microarray analysis of neutrophils infiltrating papillomas and cSCC or surrounding control skin. (A)** Genes encoding transcription factors. **(B)** Genes associated with neutrophil granules. Log<sub>2</sub> intensity is associated with a graduation from blue (low expression) to red (high expression).

**Supplementary Figure S6. Frequencies of NK and T cell subsets in mSC338-bearing mice. (A)** Frequencies of CD8<sup>+</sup> and CD4<sup>+</sup> T cells among CD45<sup>+</sup> cells in mSCC38 tumors from isotype control or anti-Ly6G-treated mice day 30 post-tumor graft, (n=10, mean  $\pm$  SEM from two independent experiments). **(B)** Frequencies of FoxP3<sup>+</sup> CD25<sup>+</sup> Treg among CD4<sup>+</sup> T cells in mSCC38 tumors from isotype control or anti-Ly6G-treated mice day 30 post-tumor graft., (n=15, mean  $\pm$  SEM from three independent experiments). **(C)** Frequencies of CD3<sup>-</sup> NKp46<sup>+</sup> NK cells among CD45<sup>+</sup> cells in mSCC38 tumors from isotype control or anti-Ly6G-treated mice day 30 post-tumor graft., (n=20, mean  $\pm$  SEM from three independent experiments). *P* value ns, Mann-Whitney *U* test.

**Supplementary Figure S7. Flow cytometry cell surface expression of selected markers on TAN from mSC338-bearing mice. (A)** Histogram overlays. **(B)** MFI of activation markers, (n=5, mean  $\pm$  SD), \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, \*\*\*\**P*<0.0001 Kruskal-Wallis test.

**Figure 1**

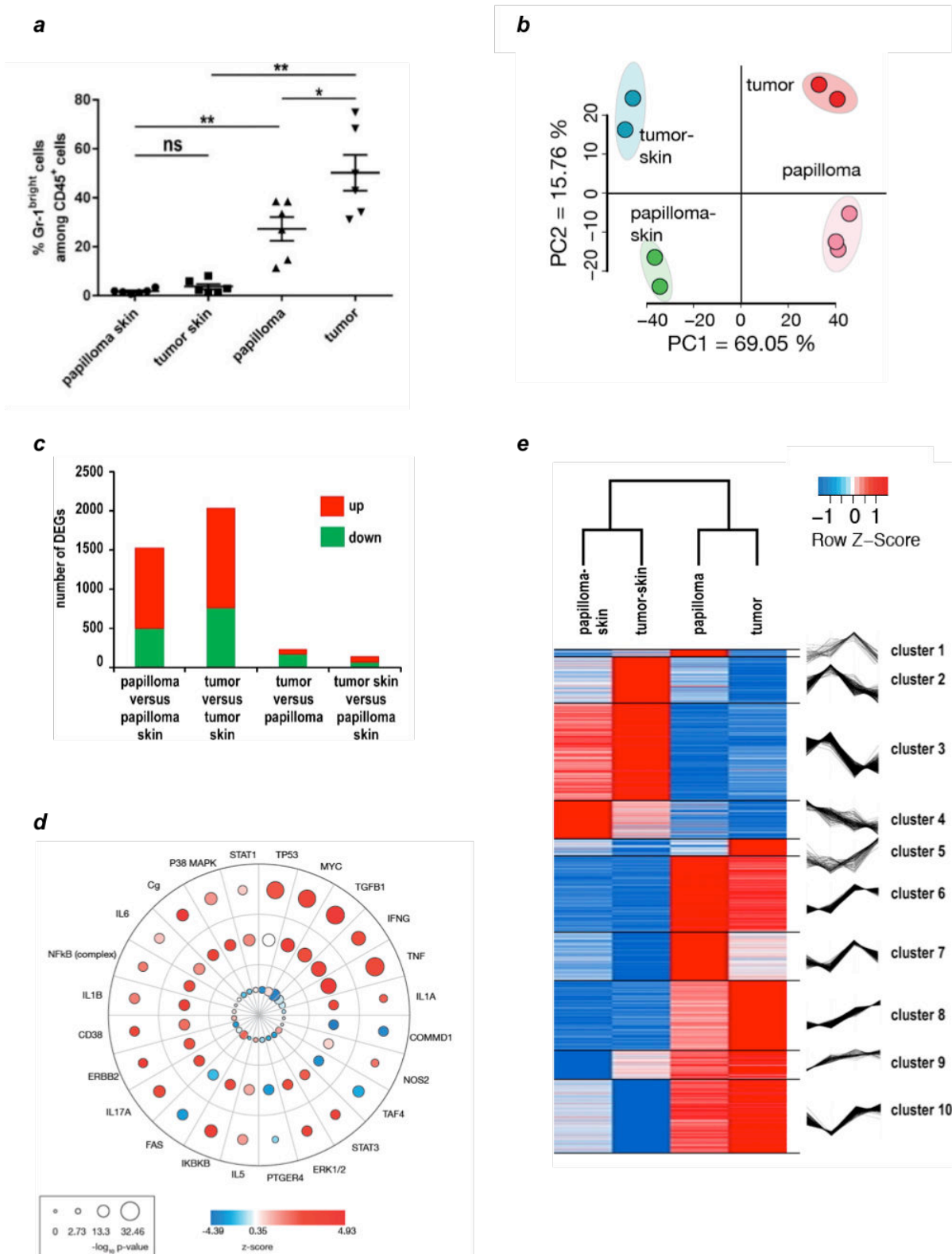


Figure 2

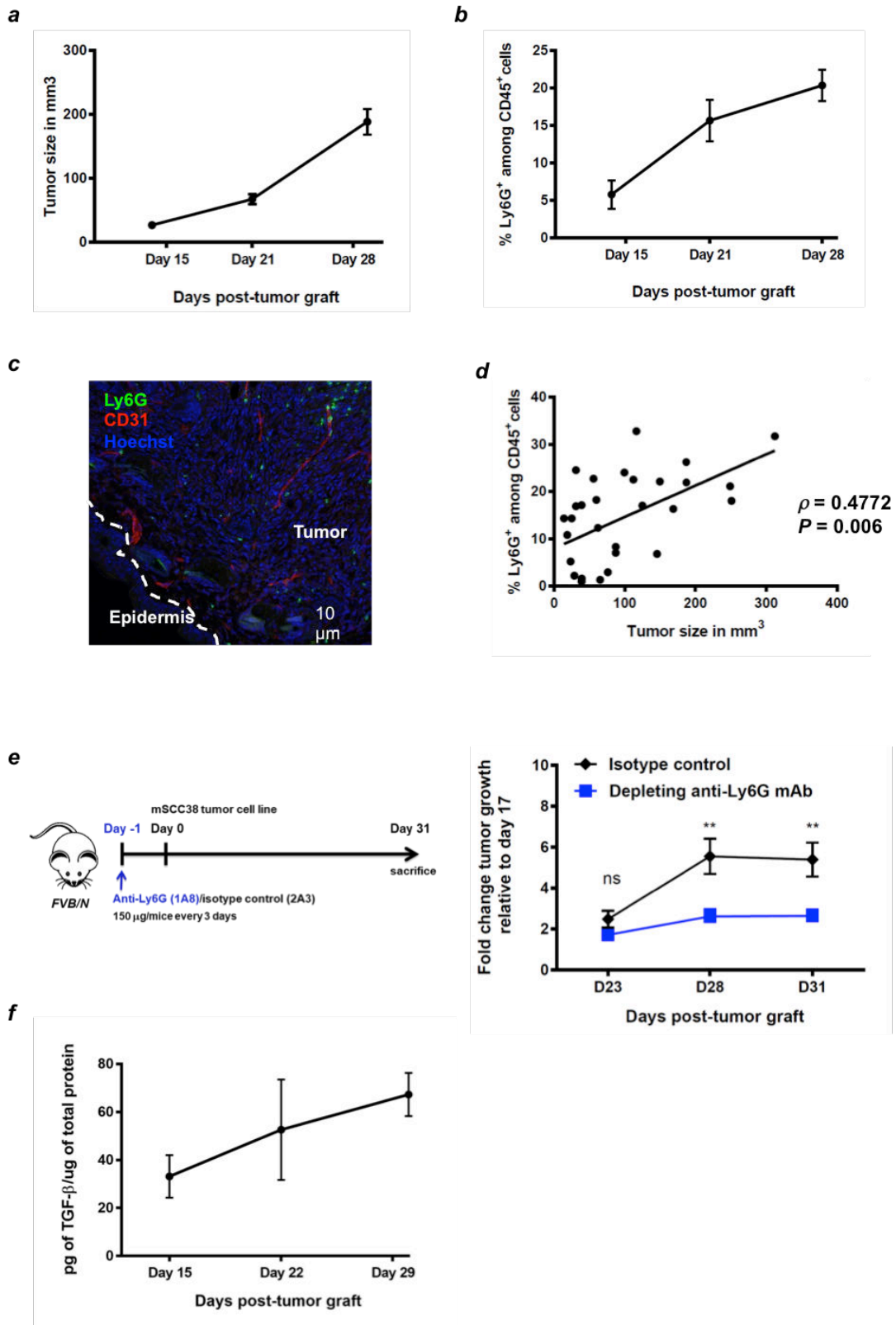


Figure 3

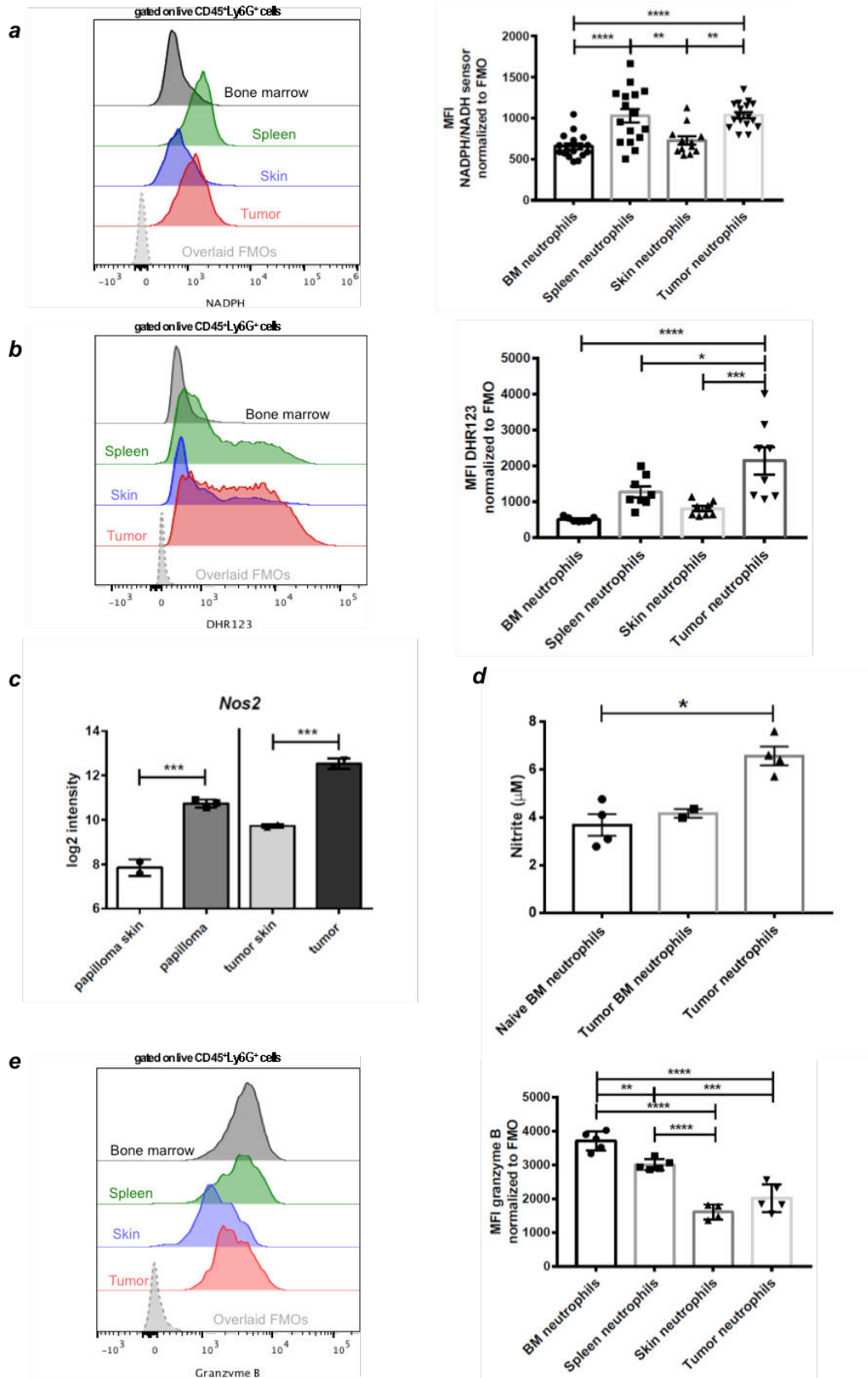
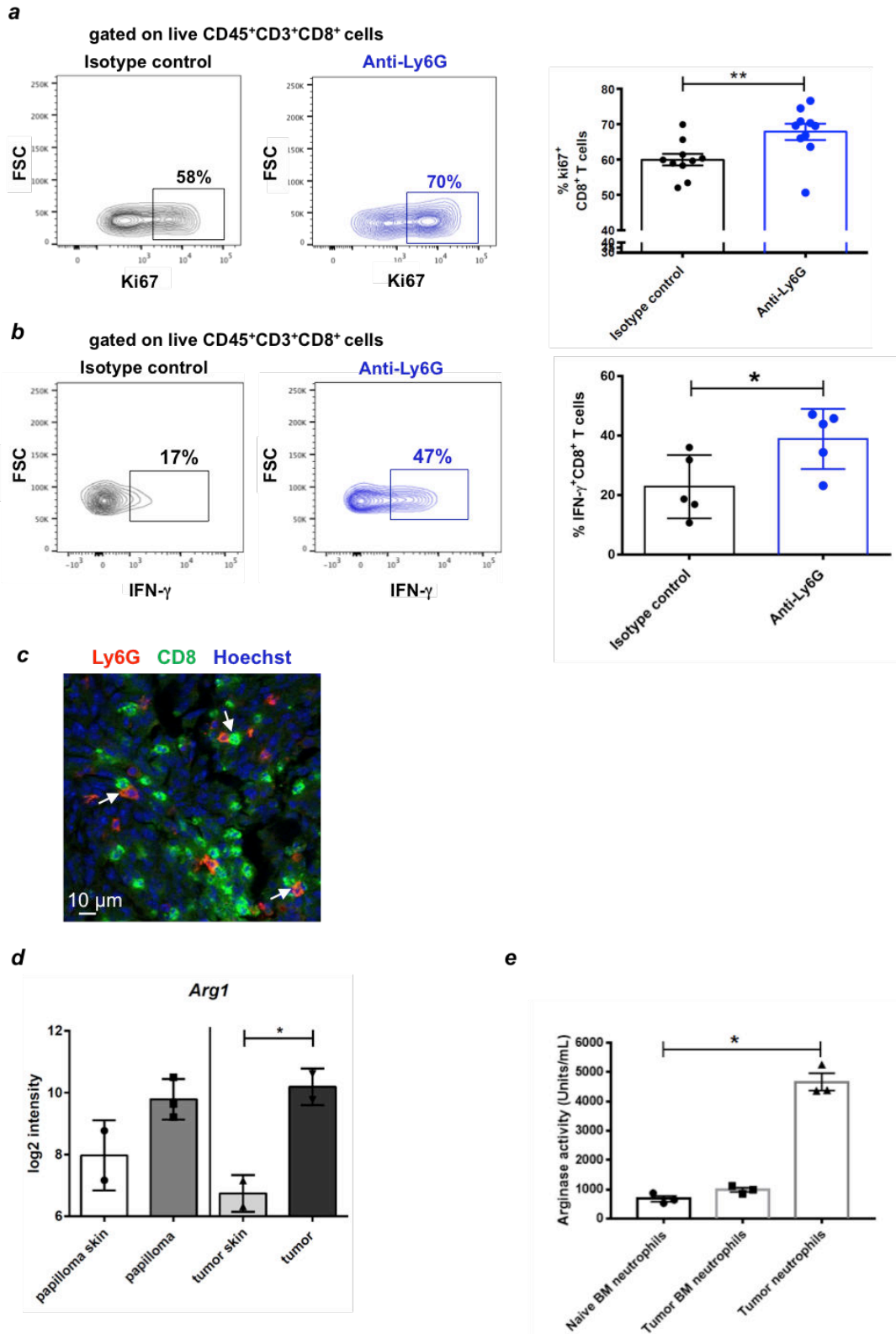
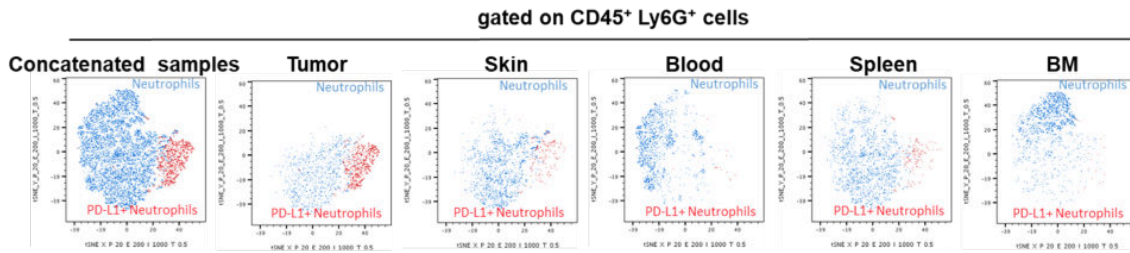


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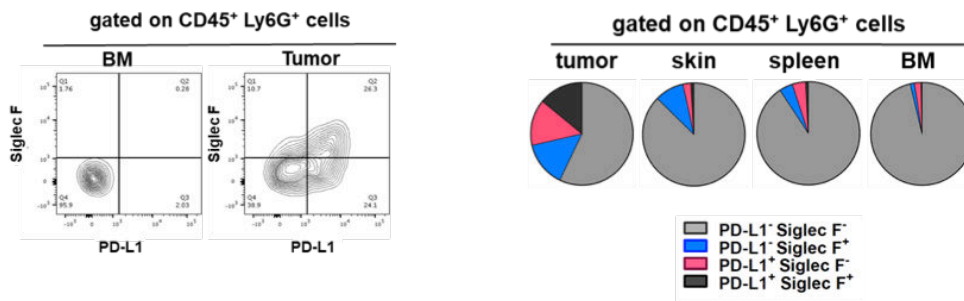


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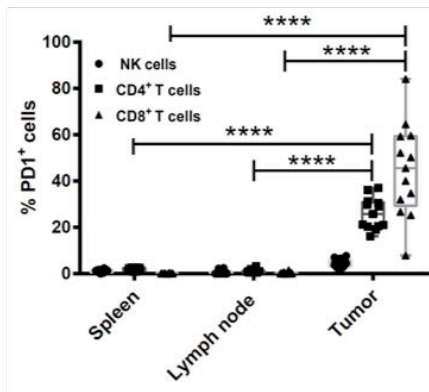
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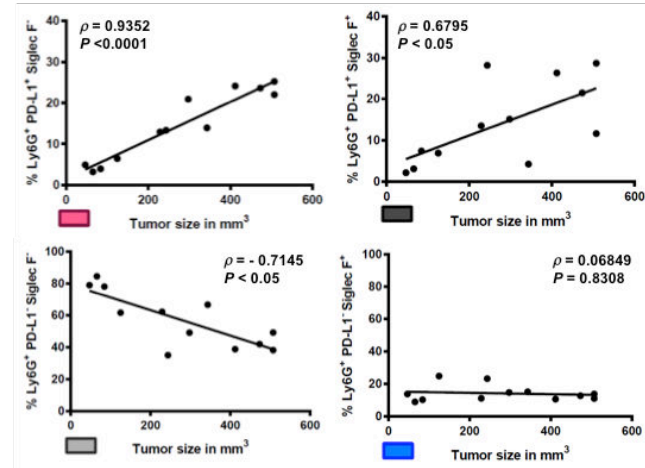
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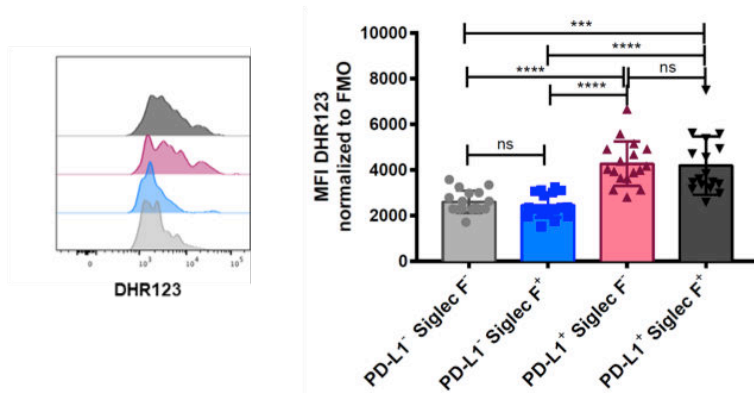
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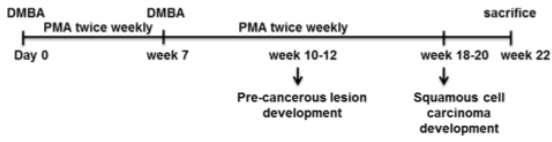


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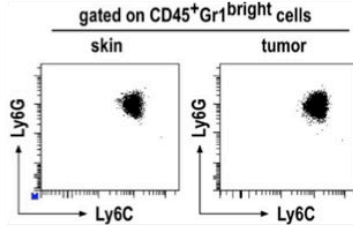


## Supplementary Figure 1

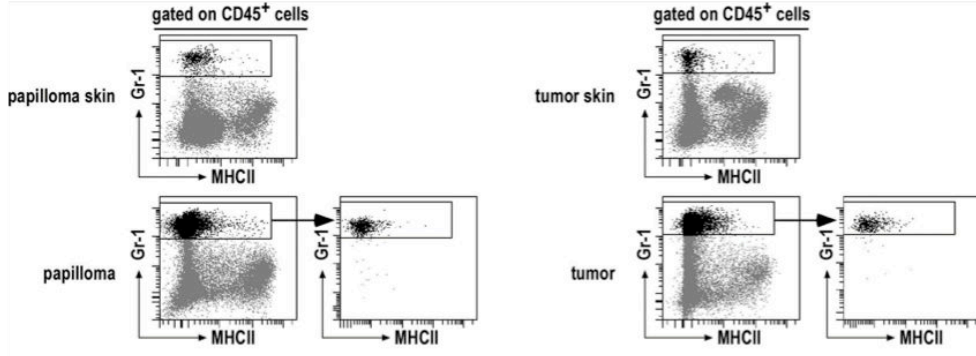
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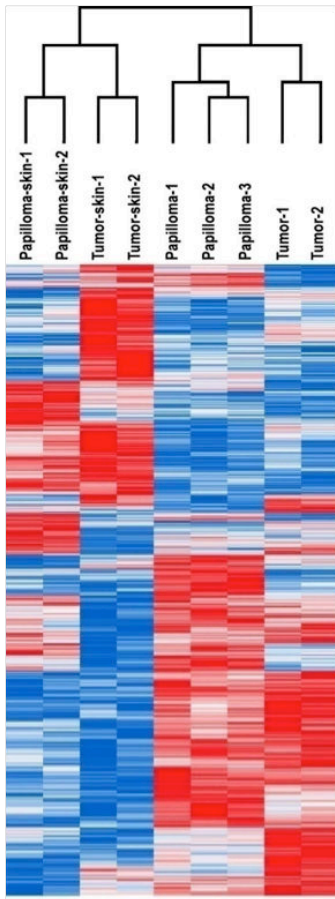
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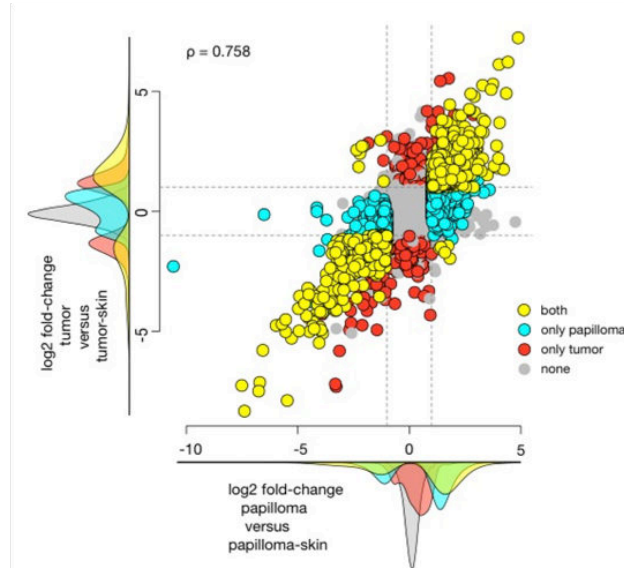
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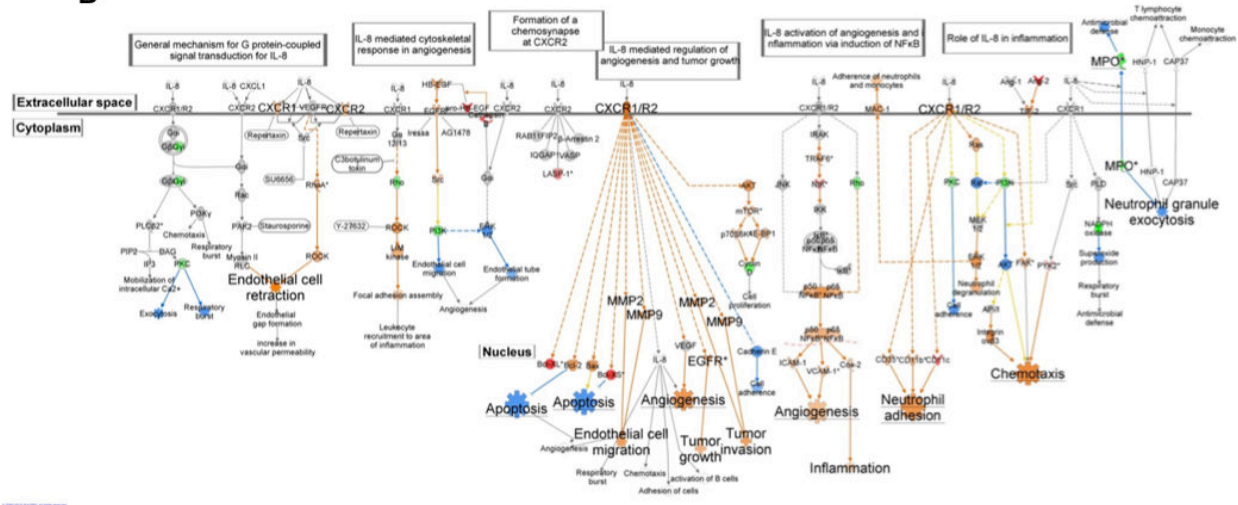


## Supplementary Figure 2

A

Canonical pathways	-Log10 (P value)			Activation z-score		
	papilloma versus papilloma skin	tumor versus tumor skin	tumor versus papilloma	papilloma versus papilloma skin	tumor versus tumor skin	tumor versus papilloma
Cholecystokinin/Gastrin-mediated Signaling	3.71	2.85	0.00	2.32	2.50	NA
IL-8 Signaling	8.31	5.52	1.97	2.92	1.26	-2.24
HMGB1 Signaling	3.87	2.46	2.87	1.21	1.21	-2.45
ILK Signaling	5.47	3.36	4.72	1.88	-0.41	-2.85
Integrin Signaling	5.81	2.53	3.69	1.63	-1.46	-3.00
Signaling by Rho Family GTPases	4.97	4.94	3.88	1.22	-2.89	-2.12
RhoA Signaling	1.01	2.38	2.11	1.00	-2.09	-2.00
Agrin Interactions at Neuromuscular Junction	2.58	3.67	2.29	0.71	-1.15	-2.00
Cardiac Hypertrophy Signaling	3.67	1.47	2.72	0.63	-1.28	-2.65
Regulation of Actin-based Motility by Rho	2.63	3.36	2.65	0.58	-2.32	-2.65
Tac Kinase Signaling	2.89	1.91	1.56	-0.28	-3.09	-2.54
Rac Signaling	0.54	1.93	Inf	-0.38	-2.67	0.00
Role of NFAT in Cardiac Hypertrophy	1.96	0.77	2.02	-0.50	-1.00	-2.24
HGF Signaling	1.72	3.56	0.00	-0.63	-2.32	NA
Leukocyte Extravasation Signaling	3.25	3.30	1.30	-0.77	-2.40	-2.24
LXR/RXR Activation	3.33	2.42	1.47	-1.73	-2.94	0.00
PPAR Signaling	4.66	3.20	0.21	-2.32	-2.59	NA
Cell Cycle: G1/S Checkpoint Regulation	0.64	1.52	0.87	NA	2.12	NA
RhoGDI Signaling	4.56	3.48	3.43	-1.41	0.85	2.45

B



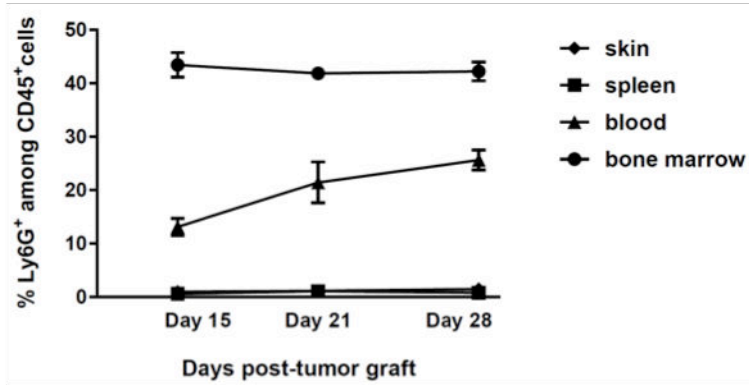
### Supplementary Figure 3

## Gene cluster GO-enrichment analyses

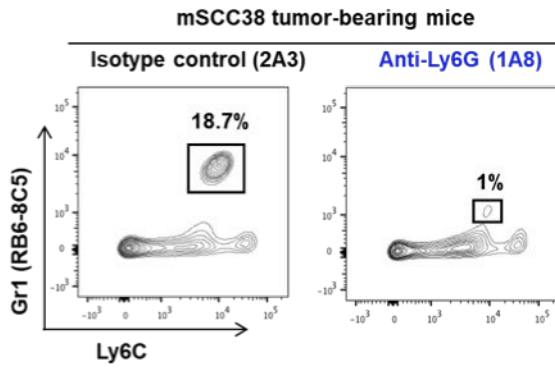
Cluster	PantherPathways		Biological Process		Protein Class		Cellular Component	
	Class	Fold enrichment (p-value)	Class	Fold enrichment (p-value)	Class	Fold enrichment (p-value)	Class	Fold enrichment (p-value)
Cluster 1			endocytosis	> 5	extracellular matrix structural protein	> 5		
Cluster 2	Cytoskeletal regulation by Rho GTPase	> 5	cellular component organization	2.99				
			cellular process	1.57				
Cluster 3	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway inflammation mediated by chemokine and cytokine signaling pathway Peritose phosphate pathway B cell activation	> 5	cellular component morphogenesis	4.48				
			anatomical structure morphogenesis	3.93				
			cellular component organization or biogenesis	2.73				
			regulation of molecular function	2.29	G-protein modulator	3.73	actin cytoskeleton	3.07
Cluster 4	Inflammation mediated by chemokine and cytokine signaling pathway	> 5	regulation of catalytic activity	2.27	guanyl-nucleotide exchange factor	4.61	2.13	
			metabolic process	1.33	enzyme modulator	1.97	1.84	
Cluster 5	mesoderm development	> 5	receptor	2.46				
			defense/immunity protein	3.75				
Cluster 6	Integrin signalling pathway	> 5	cell communication	10.18	extracellular matrix protein	> 5	11.69	
			biological adhesion	> 5	signaling molecule	3.64	7.28	
Cluster 7	Integrin signalling pathway	> 5	cell-cell adhesion	> 5	extracellular matrix structural protein	> 5	5.61	
			cell adhesion	> 5	extracellular matrix linker protein	> 5	4.72	
			developmental process	2.65	surfactant	> 5	4.06	
			cellular process	1.75	cell adhesion molecule	4.26	3.52	
			ectoderm development	4.37	receptor	2.3	2.91	
			system process	2.8	membrane-bound signaling molecule	> 5	1.83	
			single-multicellular organism process	2.56	extracellular matrix glycoprotein	> 5	1.79	
			multicellular organismal process	2.56	nucleic acid binding	0.26	1.78	
			regulation of liquid surface tension	> 5	antibacterial response protein	> 5	1.43	
			cell-matrix adhesion	> 5				
			immune system process	2.58				
			homeostatic process	> 5				
			mesoderm development	3.49				
			system development	2.71				
cell-cell signaling	3.51							
macrophage activation	> 5							
nervous system development	3							
endocytosis	3.89							
response to stimulus	1.88							
receptor-mediated endocytosis	> 5							
Cluster 8	Glycolysis	> 5	glycolysis	2.61	intermediate filament cytoskeleton	> 5	1.35	
Cluster 9	Glycolysis	> 5	glycolysis	2.40	cytosine protease inhibitor	> 5	2.48	
			monosaccharide metabolic process	> 5				
Cluster 10	CCR6 signaling map	> 5	cellular component biogenesis	4.2				
			metabolic process	1.32				
			nitrogen compound metabolic process	2.26				
			cellular component organization or biogenesis	2.08				

## Supplementary Figure 4

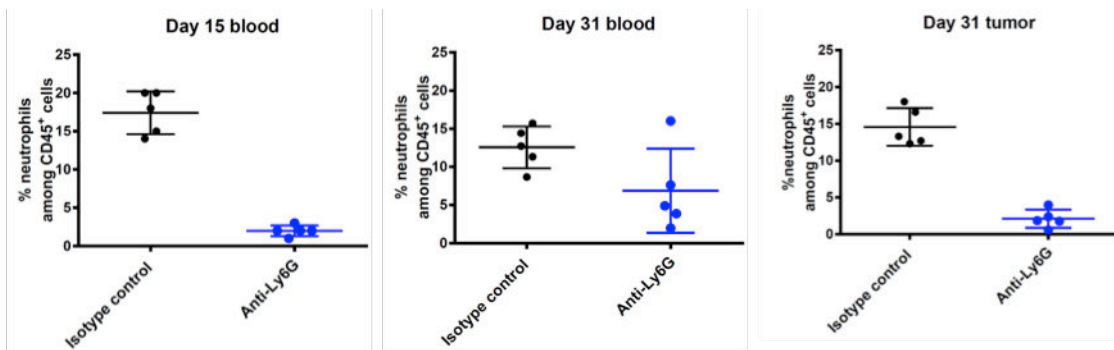
**A**



**B**

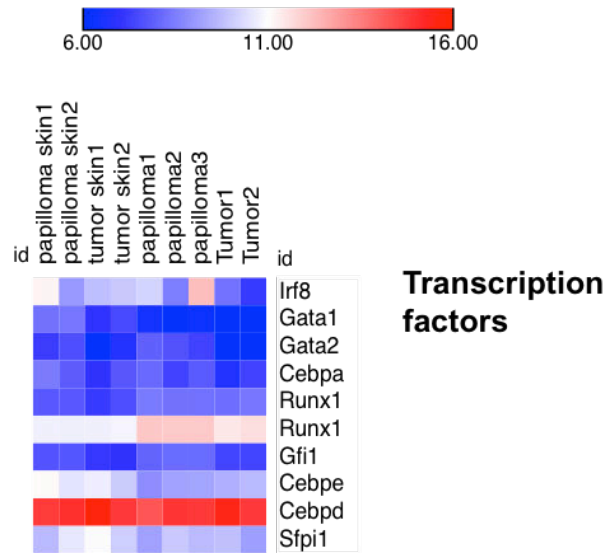


**C**

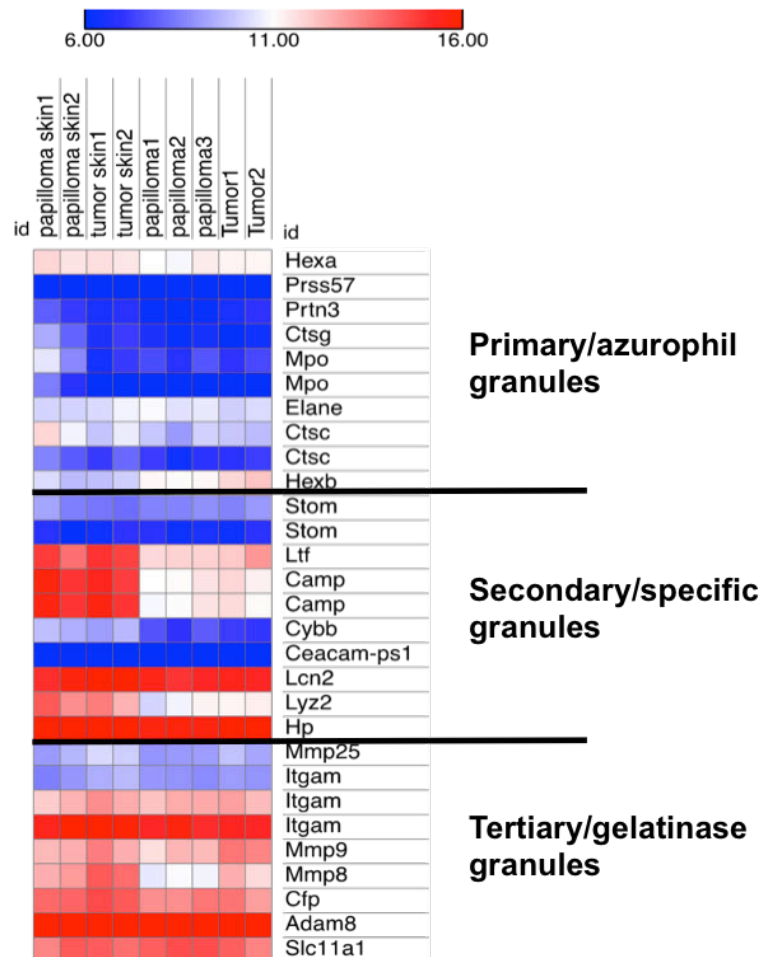


Supplementary Figure 5

A

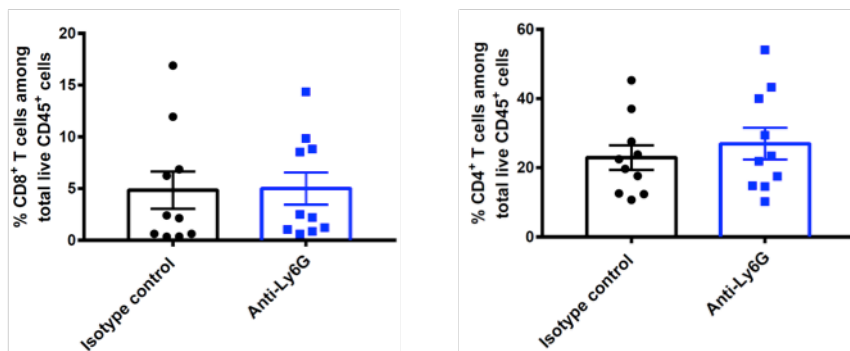


B

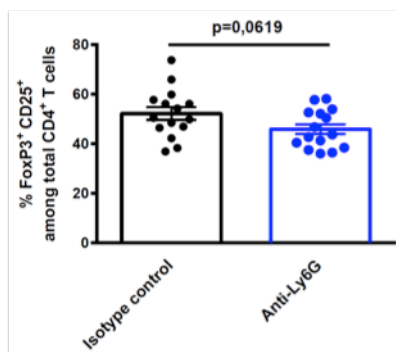


## Supplementary Figure 6

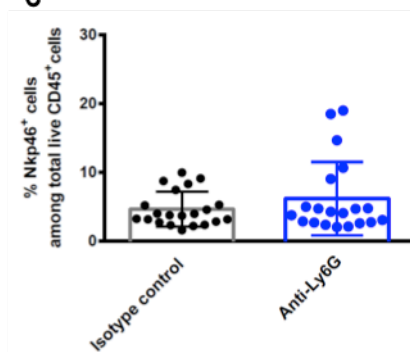
A



B



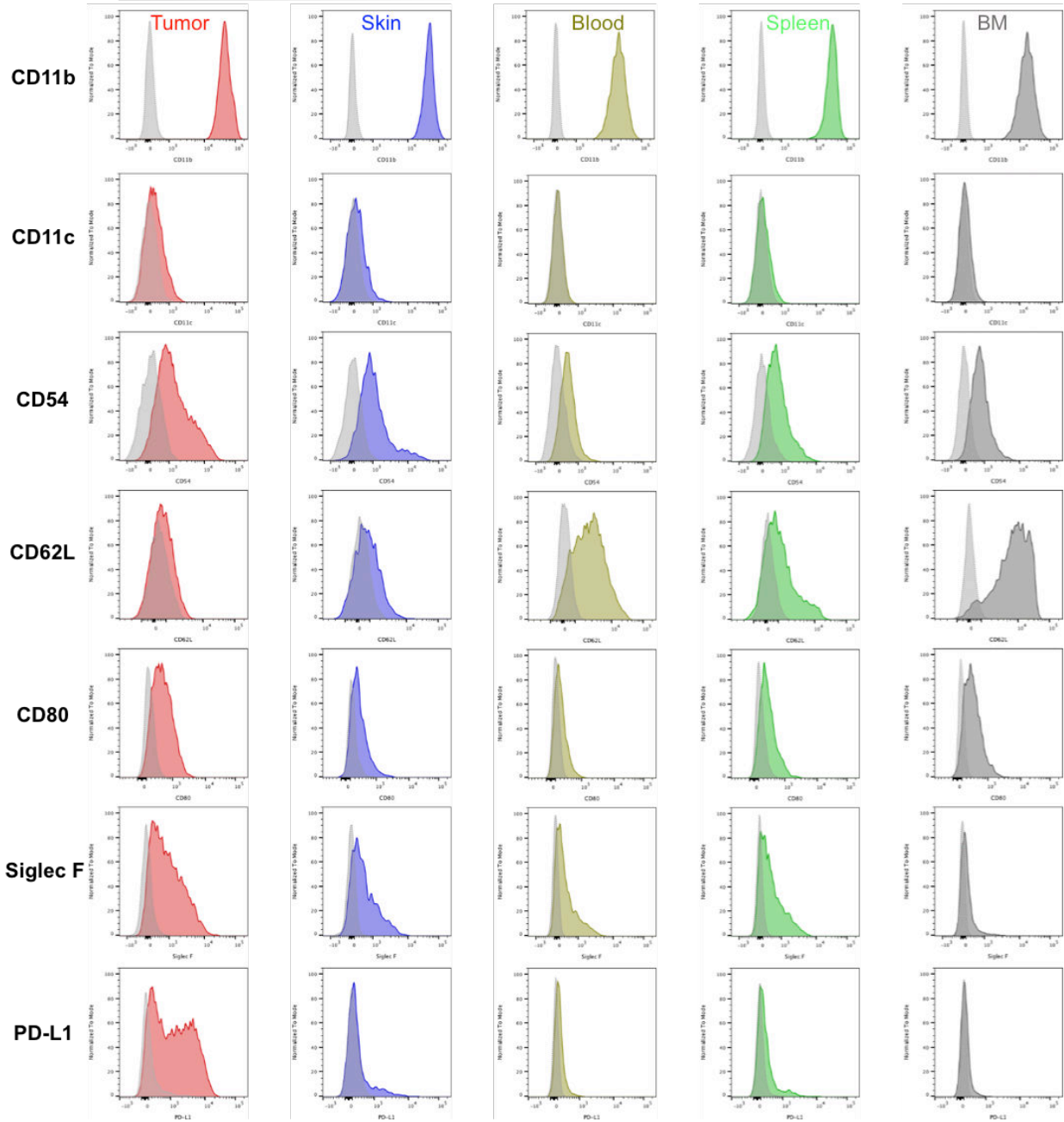
C



# Supplementary Figure 7

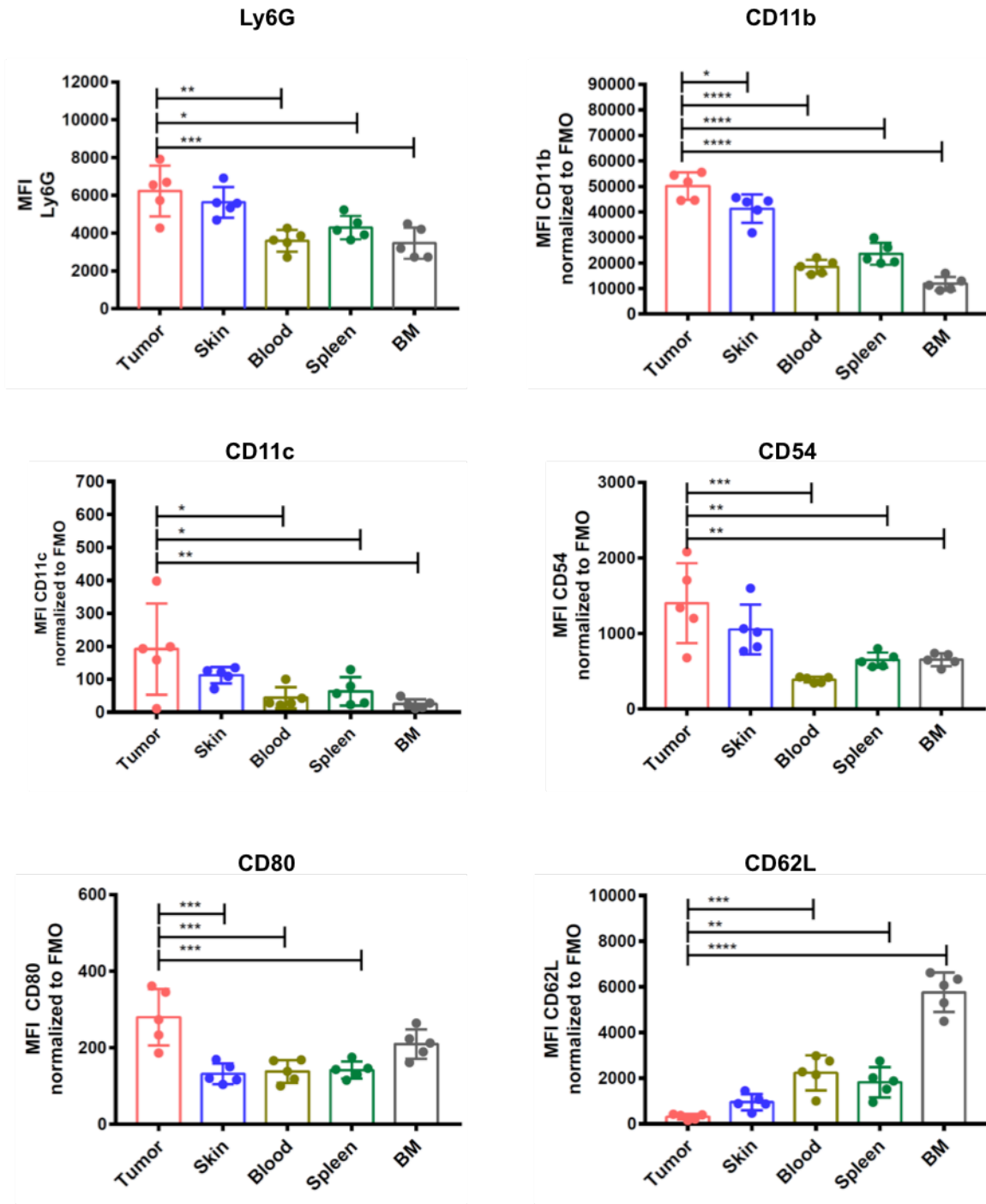
A

gated on live CD45<sup>+</sup> Ly6G<sup>+</sup>



## Supplementary Figure 7

B



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# **DISCUSSION**

## Relevance of neutrophils in cancer

Neutrophils have long been viewed as cells critical for early defense to microbial infections. More recently, they have also been found infiltrating tumors and modulating tumor growth. The question of the relevance of neutrophils in cancer is being addressed by researchers and clinicians. Our work also contributes to provide answers, notably in the context of cSCC.

### **Can neutrophil counts in blood or within tumor predict patient outcome?**

Because neutrophils are associated with inflammation, they have been found in higher numbers in the blood of patients suffering from various types of cancers. A meta-analysis by Templeton et al has shown that a high **Neutrophil-to-Lymphocyte ratio** (NLR) in blood is associated with an adverse overall survival (OS) (Templeton et al., 2014). A strong prognostic and predictive value has been attributed to NLR for human solid tumors. Pathologists, using immunohistochemical analyses on tissue-micro arrays (TMA), have also investigated the **immune infiltrates in human tumors** and whether they could predict disease outcome. Pioneer work was performed by the group of Jerome Galon (Galon et al., 2006; Pagès et al., 2005). They could show that the infiltration of T cells was associated with favorable clinical outcome. Later on, they extended their studies to all immune cell types and found that while most T cell markers were highly expressed in tumors at an early stage, they could detect an increased density of neutrophils along other cell types like macrophages, B cells and DC, at later tumor stages (Bindea et al., 2013). Gentles et al also performed a meta-analysis of gene expression datasets from 39 human solid tumors (Gentles et al., 2015). They used the CIBERSORT (Cell Type Identification By Estimating Relative Subsets Of Known RNA Transcripts) method developed by Newman et al, which is an analytical tool that uses gene expression data to estimate the abundance of cell subsets in a mixed cell population (Newman et al., 2015). This is based on gene signatures defined for each cell subset. This extensive study analyzed the abundance of each immune cell type in relation to clinical outcome. The study revealed that TAN are associated with the worst clinical outcomes (Gentles et al., 2015).

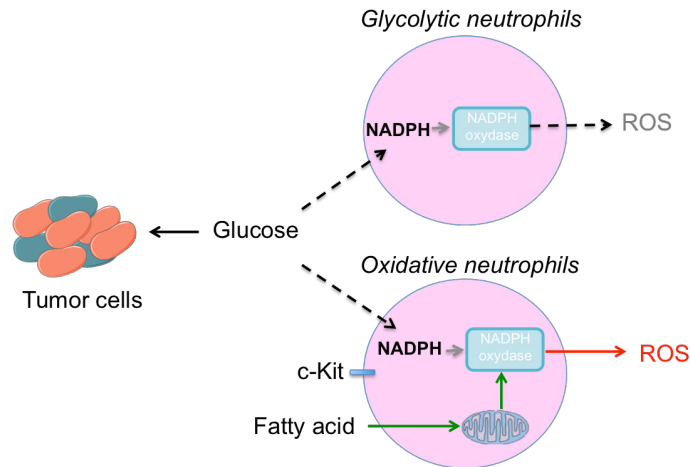
Altogether, these complementary approaches points to a tumor-promoting role for TAN. Caution must be taken because these observations are correlative but not necessarily causative. In addition, the identification of TAN relies on the use of makers that are found expressed by neutrophils but also by other immune cells (ie CD15 may be expressed by eosinophils, CD66b is expressed by basophils,...) or on markers that could be modulated within tissue type and cancer type. This adds difficulties to definitively conclude on the nature of the infiltrated cells. A limit of these studies is the time of the diagnostic which is generally late. In humans, it is therefore difficult to evaluate the role of TAN throughout tumor progression. Bindea et al detected lower level of infiltration of TAN at the early stage (Bindea et al., 2013). Eruslanov et al isolated TAN from human lung tumors at early stage and they found they were capable of stimulating T cell responses. They correlated this anti-tumoral function with early stages of cancer (Eruslanov et al., 2014b). In the same line, Mishalian et al observed that murine TANs in the grafted tumors at early stage of development were more capable to kill tumor cells and produced higher amount of oxidants than those present at the late stage. Interestingly, TANs in these models were found exclusively localized in the periphery of the tumor and only at the late stage were embedded inside the tumor (Mishalian et al., 2013). Faget et al analyzed the immune signature in both small and big lung adenocarcinomas by flow cytometry (Faget et al., 2017). TAN were highly represented in the big tumors that are also the most advanced. This suggests that TAN may be pro-tumoral at later stages but it is more difficult to conclude on the function of TAN at earlier stages. Considering the complexity of functions of neutrophils and their modulation throughout tumor progression, caution must be taken to conclude on their impact on the clinical outcome. In our two stages disease model of cSCC triggered by application of DMBA and PMA, we found an overall similar modulation of genes in neutrophils isolated from either the precancerous stage, the papillomas, or the tumors. However, we could also identify specific functions at each stage, like neutrophils involved in angiogenesis and ECM remodeling at the precancerous stage or neutrophils involved in immune suppression at the tumor stage.

## **Modulation of neutrophil behaviors within the tumor microenvironment**

### **➤ Metabolic adaptation**

In the tumor microsystem, the environmental conditions such as pH, nutrient and oxygen content affect and are affected by cancer cells and other cell types. Fast growth of tumor often accompanied by a poor vasculature system decreases oxygen level in the interior of tumors. Hypoxic microenvironment generated by a low level of oxygen leads to many changes in cellular behaviors for example metabolic shift notably in cancer cells toward anaerobic mitochondrial energy production. Accumulation of the end-products of this latter, including lactic acid and CO<sub>2</sub> leads to a decrease of intratumoral pH, which is another stress factor that can shape cellular behaviors. This metabolic adaptation is also observed in immune cells with hypoxia inducible factors (HIF), being one of the main actors (Samanta and Semenza et al., 2018). These tumor-induced modifications of myeloid cell metabolism contribute to the reprogramming of their functions (Porta et al., 2018). Indeed, MDSCs exhibited an enhanced fatty acid oxidation and mitochondrial function, associated with immunosuppressive functions (Hossain et al., 2015). Among MDSCs, PMN-MDSCs or TAN, which were believed to exclusively depend on glucose, were reported to metabolize fatty acid to maintain intracellular level of NADPH, thus their capacity to produce reactive oxygen species (ROS) through NADPH oxidase (Figure 13). (Rice et al., 2018).

**Figure 13.** Metabolic adaptation of TANs in tumor microenvironment adapted from (Rice et al., 2018) showing that in a glucose-limited environment, neutrophils engaged in oxidative mitochondrial metabolism



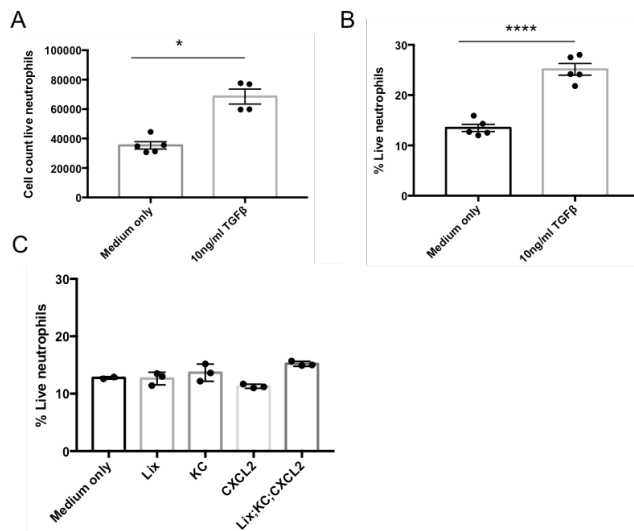
### ➤ Survival

A role for neutrophils in cancer was not considered for a long time because of their reported short half-life. They are believed to undergo constitutive apoptosis in order to maintain the homeostasis in the peripheral. However, it was reported that hypoxia could prolong neutrophil survival in a HIF-1 $\alpha$ -mediated manner (Hannah et al., 1995; Walmsley et al., 2005). Many studies started to question this issue and it is now accepted that neutrophils within the tumor microenvironment live longer (Coffelt et al., 2016).

TGF- $\beta$  is known to polarize neutrophils towards a pro-tumoral phenotype and is also able to increase their survival (Fridlender et al., 2009; Lagraoui and Gagnon et al., 1997). From our transcriptomic analysis, we identified IL-8 signaling pathway as the top canonical pathway. Triggering through CXCR1 and CXCR2 resulted in the activation of genes that were involved in neutrophil survival. These results suggest that in cSCC, neutrophils from papillomas and tumors survive longer than neutrophils found in their surrounding skin. To go further into the mechanism, we tested the effect of TGF- $\beta$ . Neutrophils isolated from BM of naïve mice were cultured *in vitro* in the presence of 10 ng/ml TGF- $\beta$ . We observed that a greater proportion stayed alive (**Figure 14 A and B**). By contrast, we tested the effect of three mouse homologues

of IL-8 (Lix, KC and CXCL2) either alone or in combination at 10 ng/ml. No increase of survival was measured (**Figure 14 C**). Further experiments will be set up to decipher the mechanisms triggering increased survival.

**Figure 14. TGF- $\beta$  enhances neutrophil survival.** Bone marrow cells of naïve FVB/N mice were enriched in neutrophils using Histopaque concentration gradient method and were next flow cytometry-sorted using neutrophil specific marker Ly6G. 500 000 of purified neutrophils were cultured in 200  $\mu$ l in a 96 well plate with or without cytokine for 48hr. Cell survival was assessed by using Caspase-3/7 Green Flow cytometry assay kit. **A.** Absolute cell count of live neutrophils after culture with 10ng/ml TGF- $\beta$ . **B.** Proportion of live neutrophils after culture with 10ng/ml TGF- $\beta$ . (*neutrophils purified from 2-3 mice, triplicates of two independent experiments*). **C.** Proportion of live neutrophils after culture with 10ng/ml Lix, KC, CXCL2 or Lix/KC/CXCL2. (*neutrophils purified from 2 mice, triplicates of one independent experiment*).



### ➤ Polarization into anti-tumoral and pro-tumoral neutrophils

Both anti- and pro-tumoral functions have been described. Fridlender et al demonstrated that TGF- $\beta$  in the tumor microenvironment polarized neutrophils towards a N2 pro-tumoral phenotype (Fridlender et al., 2009). The blockade of TGF- $\beta$  promoted N1 anti-tumoral neutrophils. Polarization into N1/N2, by similarity to M1/M2 macrophages, has its limitation.

While the specificity of both subsets is well described in mouse models of cancers, it is more difficult to provide clear evidence in humans. Also, the discrimination between N1 and N2 neutrophils might not be simple. It has been suggested that the difference depends on the level of activation. Indeed, anti-tumoral functions of neutrophils are generally measured in situations where neutrophils are activated (Houghton, 2010). In the situation of sepsis, the level of activation is so high that it is detrimental to the patients. By contrast, in the tumor microenvironment, neutrophils may be poorly activated. This analysis is consistent with the observation that N1 and N2 neutrophils produce ROS and NO but that N1 are producing much more. In our study using the mSCC38 engraftment, we compared TAN with neutrophils from the skin, spleen, blood and bone marrow. We found that TAN and neutrophils in the skin shared markers of activation. Whether they would display lower levels than neutrophils with an N1 phenotype still needs to be demonstrated. So what makes a N2 protumoral may rely on its immunosuppressive functions. For this reason, they seem to be closely related to PMN-MDSC. The study of human cancers has also identified N1 neutrophils at early stages of the disease while N2 neutrophils were found at later stages (Eruslanov et al., 2014b; Faget et al., 2017). This observation is consistent with the immunoediting where the first phase is the elimination phase to which N1 can participate. Later, the tumor microenvironment condition neutrophil to polarize them into N2. Further work is needed to apprehend all the mechanisms that influence this polarization.

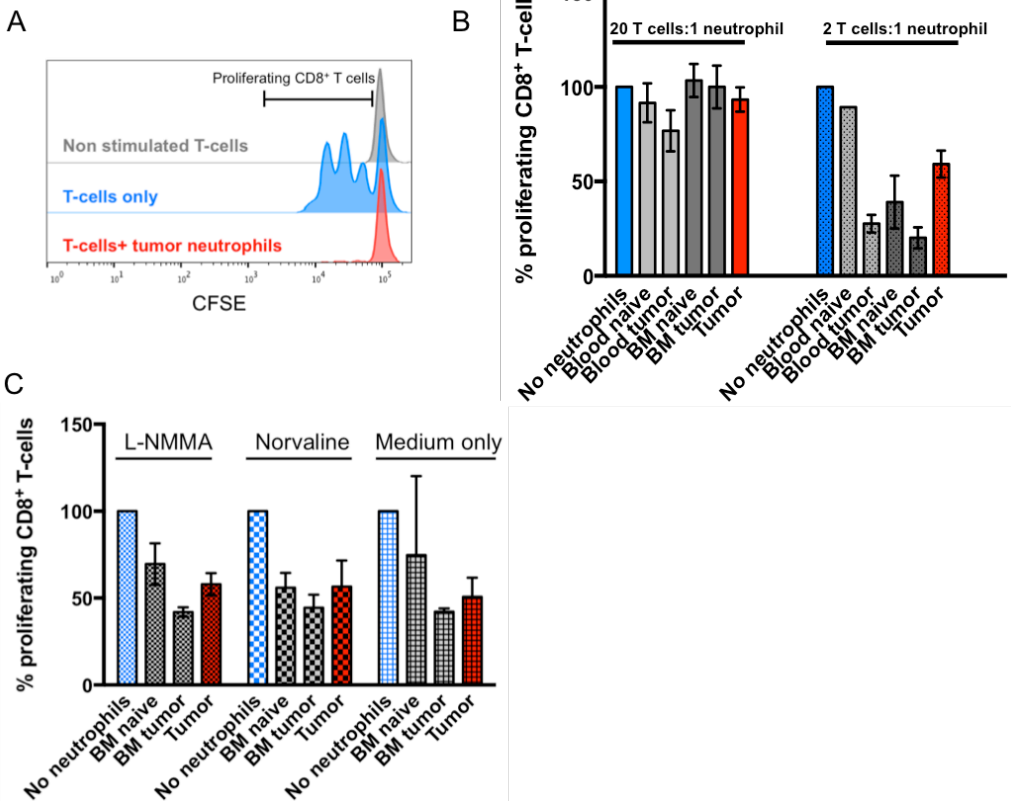
PMN-MDSC, like N2 TAN, display potent immunosuppressive functions and potent tumor promoting functions (Groth et al., 2018). Whether they are in fact the same cells is debated. MDSC has been shown to display an immature phenotype while N2 TAN do not. No specific markers have been identified that distinguish them (Bronte et al., 2016). A key assay used to demonstrate immunosuppression was the inhibition of T cell proliferation *in vitro*. However, a recent study has identified technical issues over the use of anti-CD3/CD28 coated beads and lectins that were directly polarizing neutrophils into immunosuppressor cells (Negorev et al., 2018).

In an attempt to evaluate the capacity of TAN from mSCC38 tumor to suppress T cell proliferation as compared to neutrophils from other organs in both tumor-bearing mice and

naïve mice, we also performed *in vitro* T cell proliferation. We observed that TAN could inhibit T cell proliferation in a very similar way to other controls. We were surprised by the fact that all those neutrophils could inhibit the proliferation, even those that were isolated from naïve mice (**Figure 15 A-B**). This inhibition was not abolished when either a nitric oxide synthase inhibitor (L-NMMA) or an arginase inhibitor (norvaline) were added (**Figure 15 C**). We used the anti-CD3/CD28 T cell activating microbeads that have been recently questioned by Negorev et al (Negorev et al., 2018). Therefore, it is likely that we triggered an artifactual immune suppression. To avoid this caveat, we later chose to assess immunosuppression *in vivo* by depleting neutrophils using anti-Ly6G mAb.

### **Figure 15. Inhibition of T-cell proliferation by neutrophils**

T cells were purified from lymph nodes of naïve FVB/N mice using Dynabeads Magnetic beads and stained with 4  $\mu$ M CFSE for 5 min at room temperature. Bone marrow cells from naïve and mSCC38 tumor-bearing mice were enriched in neutrophils using Histopaque concentration gradient method. Total cells from mSCC38 tumor cells were enriched in CD45<sup>+</sup> cells using Miltenyi microbead system. Neutrophils were flow cytometry-sorted from these enriched fractions and blood using neutrophil specific marker Ly6G. T cells and neutrophils were cocultured in DMEM medium supplemented with 10% FCS,  $\beta$ -mercaptoethanol at 37 °c for 60hr. **A.** Histogram representative of CFSE staining in T cells after coculture. **B.** Quantification of percentage of proliferating T cells after culture with TAN, blood and BM neutrophils. **C.** Quantification of percentage of proliferating T-cells after culture with TAN in the presence of L-NMMA or Norvaline or only medium. **(B-C)** Data from all the conditions were normalized against the percentage of CFSE<sup>+</sup> T cells incubated alone, which was considered to be 100%. (*mean  $\pm$  SEM, pool of 10 mice, 3 independent experiments, each of which were performed in triplicate*)



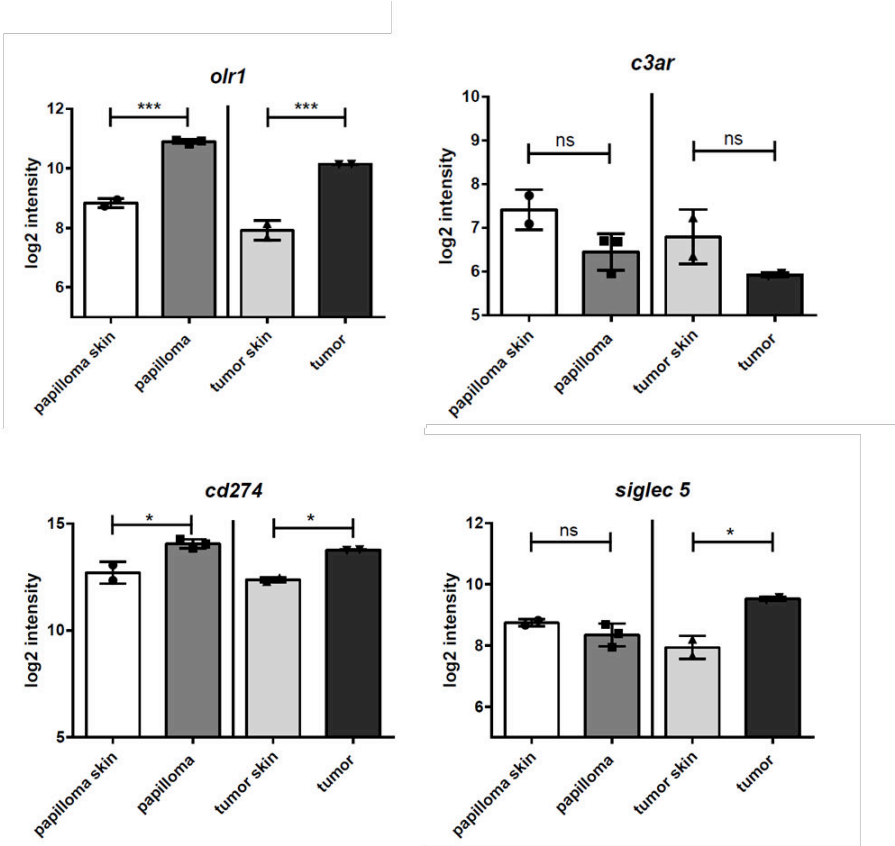
## Characterization of protumoral marker in TAN

Several pro-tumoral markers of TANs have been published in different types of tumors including LOX1 (encoded by *olr1*), Siglec F (encoded by *siglec5*), C3aR (encoded by *c3ar*) and PD-L1 (encoded by *cd274*) (Condamine et al., 2016; Engblom et al., 2017; Guglietta et al., 2016; He et al., 2015). Because we identified a pro-tumoral role of TAN in cSCC, it was relevant to monitor their expression in our models. The reported Lox1 molecule could not be tested as no anti-LOX1 mAb recognizing the mouse molecule is available. But we found the transcripts significantly upregulated in tumors or papillomas compared to their surrounding skin control (**Figure 16**). We did not test C3aR as it was not upregulated in tumors or papillomas compared to their surrounding skin control (**Figure 16**). By contrast, we assessed the level of expression of Siglec F and PD-L1. For both genes, transcripts were significantly upregulated in tumors compared to skin (**Figure 16**). When we stained TAN, we found a significant increase of expression of Siglec F and PD-L1, either alone or together. These observations are novel. Siglec

F was described as a pro-tumoral marker for TAN in lung adenocarcinomas (Engblom et al., 2017). Siglec F<sup>high</sup> TAN exhibited a gene expression profile associated with protumoral functions such as angiogenesis, myeloid cell differentiation and recruitment, extracellular matrix remodeling, suppression of T cell responses, and tumor cell proliferation and growth. Once adoptively transferred to tumor-bearing mice, they accelerated tumor growth compared to their control counterpart Siglec F<sup>low</sup> neutrophils. They also produced a higher level of ROS. In our cSCC models, we only found higher expression of Siglec F on TAN but this was not correlated with tumor growth and also not associated with higher ROS production. Therefore, its role remains unclear in cSCC.

PD-L1 has previously been found expressed in hepatocellular carcinoma (He et al., 2015), colon cancer (Lu et al., 2016), and in lung cancer (Yamauchi et al., 2018). We now need to assess PD-L1 expression on TAN in human cSCC. In addition, we found that PD-L1 expression was associated with increased ROS production and positively correlated with tumor growth. This is also the first report of such link. We now need to assess which other functions of TAN are associated with PD-L1 expression. To do so, we aim to perform some RNA Seq analysis of flow cytometry cell-sorted PD-L1<sup>+</sup> and PD-L1<sup>-</sup> TAN. Expression of PD1 is also highly upregulated on CD8<sup>+</sup> T cells and to a lesser extent on CD4<sup>+</sup> T cells. Therefore, a role for PD-L1/PD1 interaction in the immunosuppression of anti-tumoral CD8<sup>+</sup> T cells is likely. To confirm this in vivo, we will try to perform adoptive transfer of cell-sorted PD-L1<sup>+</sup> and PD-L1<sup>-</sup> TAN together with mSCC38 or may consider knocking down PD-L1 specifically in neutrophils. Interestingly, a recent clinical trial using Cemiplimab, an anti-PD1 mAb in advanced cSCC showed good response in half of the patients (Migden et al., 2018). It is also worth noting that PD-1 inhibitors were recently approved by US FDA for head and neck SCCs during or after platinum chemotherapy (Argiris et al., 2017; Bauml et al., 2017). Several case reports of cSCC showed promising results in response to immune checkpoint therapy. Patients with advanced cSCC are reported to be responsive to anti-PD-1 antibody (nivolumab and pembrolizumab) (Degache et al., 2018; Deinlein et al., 2017; Stevenson et al., 2017; Winkler et al., 2017).

Figure 16. Gene expression of *lox1* (*Olr1*), *c3ar*, *cd274* and *siglec5* in papilloma and tumor compared to their respective skin controls retrieved from microarray analysis in DMBA/PMA model.





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