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THE PROTECTIVE ROLE OF PTX3 IN MOUSE SKIN CARCINOGENESIS

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Abstract

The implication of the innate immune system on inflammatory carcinogenesis is a central topic in tumor biology. The humoral pattern recognition molecule PTX3 plays a fundamental role in the modulation of inflammation by regulating Complement cascade and P-selectin dependent neutrophil recruitment. Available information in human and murine 3-MCA induced sarcomas suggests a protective role of PTX3 in cancer-related inflammation. In this study we showed that PTX3-deficient mice were more susceptible to DMBA/TPA chemically-induced epithelial skin cancer than PTX3-competent mice, in term of incidence, multiplicity of papillomas and number of lesions evolving to skin carcinomas, suggesting a more aggressive behavior of $PTX3^{-/-}$ tumors. In the skin, PTX3 was strongly produced during the acute phase of carcinogenesis by infiltrating macrophages, neutrophils, and vessels. Immunohistochemical investigation of papillomas showed low presence of PTX3 in the extracellular matrix. The deficiency of PTX3 was associated with increased cancer-related inflammation in term of neutrophil infiltration, higher production of pro-inflammatory cytokines and chemokines, increased C3 and IgG deposition. In the effort to define the molecular mechanisms underlying this phenotype, we observed that P-selectin deficiency and in vivo neutrophil depletion reverted the tumor susceptibility of $Ptx3^{-/-}$ mice. All together, these results provide evidence that PTX3 is locally produced and plays a protective role in epithelial skin carcinogenesis acting as an extrinsic oncosuppressor. The mechanism of PTX3-mediated protection is explained by modulation of cancer-related inflammation regulating P-selectin dependent neutrophil recruitment and Complement activation.

Introduction

1 The link between inflammation and cancer

1.1 Pathways that connect inflammation and cancer

In 1863, Rodolf Virchow observed the presence of leukocytes in tumor microenvironment providing the first evidence regarding the links between inflammation and cancer[1]. However, only in the last decade research activity has supported this interaction giving the basis for a new strategy of cancer prevention and treatment. Mantovani et al. summarized the multiple connections between inflammation and cancer describing two pathways: an extrinsic and an intrinsic pathway[2] (Fig. 1).

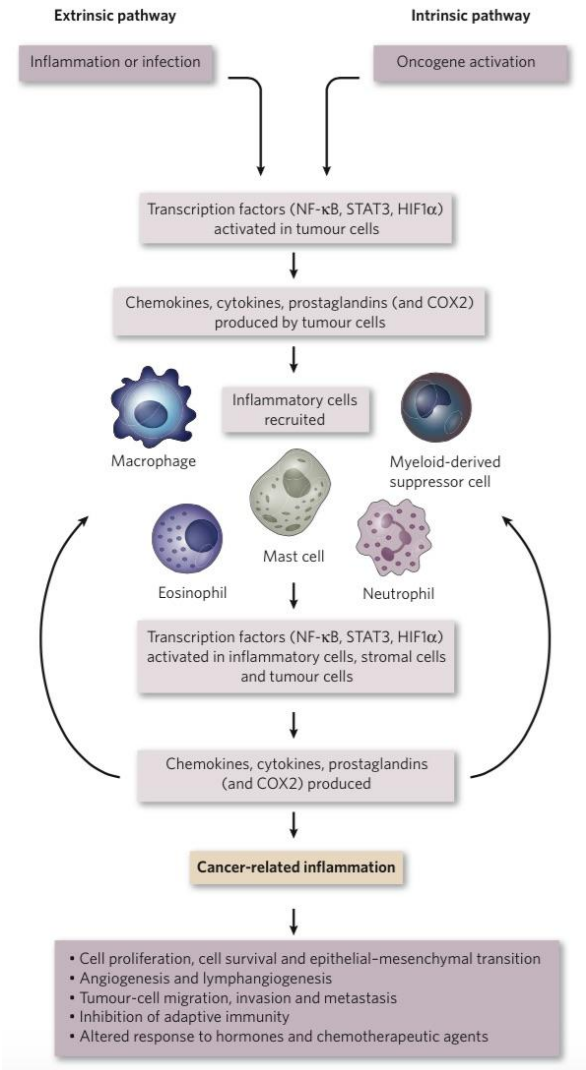

Fig. 1. Pathways that connect inflammation and cancer.
Cancer and inflammation are related by intrinsic and extrinsic pathways.
In the extrinsic pathway, chronic inflammation augments the risk to develop cancer. By contrast, the intrinsic pathway is activated by genetic events in oncogenes or tumor-suppressor genes that cause neoplasia. Transformed cells produce inflammatory mediators, thereby generating an inflammatory microenvironment also in tumors without a pre-existing inflammatory condition.
The two pathways converge, resulting in the activation of transcription factors, mainly NF-κB, STAT3 and HIF1α in inflammatory cells, and tumour cells, resulting in a cancer-related inflammatory microenvironment.
From: Cancer-related inflammation. Mantovani et al.; *Nature*. 2008.

The extrinsic pathway underlines the association of chronic inflammation with cancer risk. The majority of tumors are not hereditary but derive from environmental causes (chronic infection, obesity, tobacco smoke, silica or asbestos) that favor chronic inflammation. Prolonged infections for example with *Helicobacter pylori*, *Hepatitis B* or *C viruses*, *Papillomavirus,* increase the risk to develop gastric, hepatocellular and cervical cancer, respectively[3]. Moreover, autoimmune diseases promote tumor development, for instance, inflammatory bowel disease (IBD) is responsible for higher susceptibility to colorectal cancer[4]. Since inflammatory cell genome is not subject to gene mutations, differently from cancer cells, targeting the inflammatory microenvironment should be an unconventional strategy. Anti-inflammatory drugs, such as aspirin or cyclooxygenase 2 (COX-2) inhibitors, reduce the tumor incidence and progression of solid cancers such as colon, lung and prostate cancers[5]. However, other autoimmune disorders, for example psoriasis and rheumatoid arthritis, are not involved in tumorigenesis[2]. Chronic inflammation can contribute to carcinogenesis by increasing the proliferation of mutated cells, producing reactive oxygen species (ROS)[6] that favor genetic instability and inducing oncosuppressor-gene methylation[7] (Fig. 2).

The intrinsic pathway explains how inflammatory cells and mediators are present within tumors in absence of well-documented chronic inflammation. This pathway is activated by oncogene or tumor-suppressor gene mutations. However, transformed cells produce inflammatory mediators favoring an inflammatory microenvironment within the tumor. For example, it has been described that alterations in RET gene, common in papillary thyroid carcinoma, promoted the production of inflammatory mediators including the pro-inflammatory cytokine interlukin1β (IL-1β) and the activation of COX-2, which is involved in prostaglandin production[8]. Activation of RAS-RAF family[9,10] or MYC[11], frequently found in human and murine tumors, also promoted the production of tumor-promoting cytokines and chemokines.

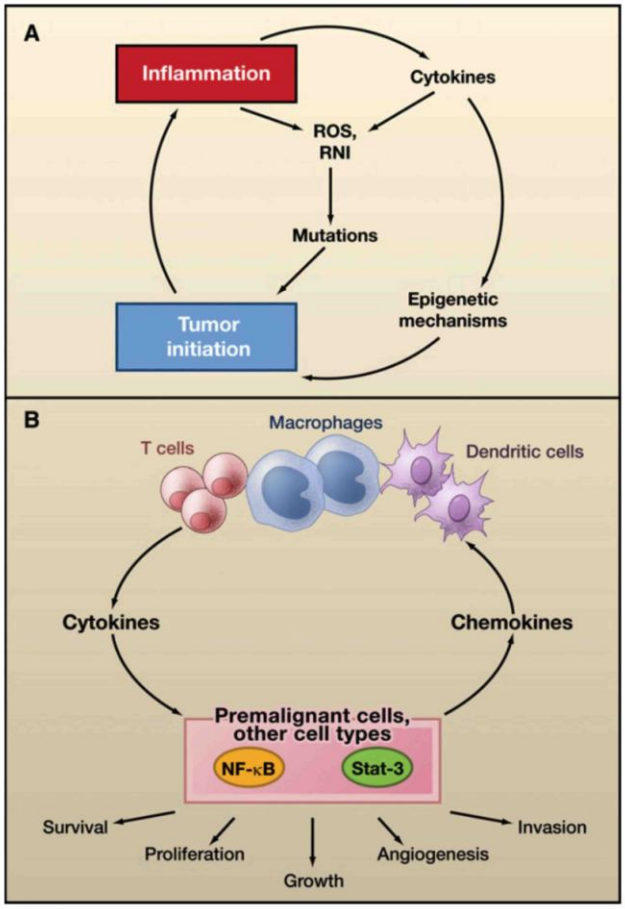

Fig. 2. (A) **Role of Inflammation in tumor initiation.** Inflammatory cells release ROS and reactive nitrogen intermediates (RNI), which increase the rate of mutations in neighboring stromal cells. In turn, cytokines produced by leukocytes favor ROS and RNI production by tumor cells. In addition, inflammation may promote epigenetic changes that favor tumor initiation.

(B) **Role of Inflammation in tumor promotion.** Cytokines produced by infiltrating leukocytes activate key transcription factors, including NF-kB or STAT3, in premalignant cells favoring tumor promotion. NF-kB and STAT3, in turn, stimulate the production of chemokines to sustain inflammation in the tumor microenvironment.

From: Immunity, inflammation, and cancer. M. Karin; *Cell*. 2010.

The intrinsic and the extrinsic pathway converge in the activation of main transcription factors such as nuclear factor-κB (NF-κB), hypoxia-inducible factor 1α (HIF1α), signal transducer and activator of transcription 3 (STAT3), in tumor cells. These factors promote in turn the production of cytokines and chemokines, which recruit inflammatory cells and favor the establishment of an inflammatory microenvironment inside the tumor[2]. Chromosomal rearrangements of *nf-kb* family members have been found in many human carcinomas and lymphomas even if NF-κB activation in tumor cells is rarely a consequence of gene alterations[12]. NF-κB acts downstream the signaling of IL-1R family and MyD88 and plays an antiapoptotic role favoring the transcription of *BCL-2* and *BCL-XL*. In addition, it promotes the transcription of angiogenic proteins such as angiopoietin and vascular endothelial growth factor (*VEGF*) and is involved in epithelial to mesenchymal transition (EMT), regulating the transcription *of TWIST* and *KISS* and matrix metalloproteases (*MMP-2* and *MMP-9*). Moreover, NF-κB upregulates pro-inflammatory

cytokines (e.g. *IL-1α*, *TNF-α*, *IL-23*) and chemokine receptors (e.g. *CXCR4*), hypoxia inducible factor 1α (*HIF1*), and other genes that control pro-tumorigenic processes in both inflammatory and tumor cells[2,3,13]. The NF-κB pathway is closely controlled at different levels. In fact, NF-κB is normally sequestered by inhibitory proteins, IκBs, in the cytoplasm of unstimulated cells avoiding the translocation into the nucleus. Upon NF-κB–activating stimuli, IκB kinase (IKK) activation, depending on its phosphorylation, promotes IκB phosphorylation and degradation, allowing NF-κB to translocate into the nucleus. A variety of other signaling events, such as the phosphorylation of IKK, the promotion of IκB synthesis, the processing of NF-κB precursors and the phosphorylation of NF-κB provide additional mechanisms that regulate NF-κB activity. It has been described that TIR8, also known as single Ig IL-1 receptor (IL-R)-related molecule, SIGIRR, competed with MyD88 and IRAK recruitment at the TIR domain of TLR/IL-1R thus dampening the signaling pathway leading to NF-kB activation[14]. TIR8 played a protective role in murine models of colitis-associated cancer by modulating bacteria-induced TLR signaling and consequently production of inflammatory mediators[15]. However different roles of NF-κB have been described in different tumors such as its inhibiting role in murine models of skin carcinogenesis and hepatocellular carcinoma[6,16].

All this evidence suggests the presence of inflammatory cells, chemokines and cytokines in the tumor microenvironment in both animal models and humans from the earliest stages of tumor development. Furthermore, the targeting of leukocytes, inflammatory mediators, including chemokines and cytokines or transcription factors involved in inflammation (like NF-κB and STAT3) may represent a new therapeutic strategy in cancer.

1.2 Balance between immune-surveillance and tumor-promoting inflammation

At the end of the nineteenth century, the observation of tumor regression in cancer patients with postoperative infections led Coley et al. to administer a mixture of toxins into the tumors,

obtaining higher survival of individuals with advanced cancer[17]. Now it is well known that, as for pathogens, the immune system can recognize specific tumor-antigens and eliminate transformed cells through an extrinsic tumor-suppressor mechanism, known as immunosurveillance[18]. The interest in the field of tumor immunosurveillance grew in the last decade after the observation that interferon γ (IFN-γ) or recombination activating gene-2 (RAG-2) deficient mice had increased incidence than wild-type (WT) mice to 3-methylcholantrene (3-MCA)-induced sarcoma[19]. However, $Rag2^{-/-}$ sarcomas implanted in WT mice were more rejected, compared to WT sarcomas, indicating an higher immunogenic potential of tumors growing in immune-compromised animals[19]. Many observations suggest that tumor cells influence the immune system to avoid immune recognition and favor tumor-promoting inflammation. These data have led to develop a new concept defined tumor immunoediting, which explains the dynamic process of tumor immune-surveillance including elimination, equilibrium and escape phases[18] (Fig. 3). In this regard, the injection of tumor cells cannot be considered a good model that mimics the first phase of carcinogenesis because it has already escaped cancer immunity, differently from carcinogen-induced models of cancer. The most studied murine models of carcinogen-induced cancer are 3-MCA-induced fibrosarcoma and DMBA (7,12-di-methylbenz(a)anthracene) and TPA (12-O-tetradecanoyl-phorbol-13-acetate) induced cutaneous squamous cell carcinoma (SCC). In the 3-MCA model, tumor-promoting inflammation and antitumor immunity overlap: in the first stage of carcinogenesis, inflammatory cells and molecules contribute to tumor induction until an antitumor immune system develops to eliminate transformed cells. Mice lacking for cells of adaptive immunity such as αβ or γδ T-lymphocytes, and of innate immunity such as innate-like T cells, NKT cells, NK cells or eosinophils showed increased tumor susceptibility to 3-MCA-induced sarcoma[18]. Moreover, high number of soluble immune mediators (perforine[20], TRAIL[21]) and cytokines (IL-12[22], IFN-αβ[23], IFN-γ[20]) were also involved in the first phase of tumor elimination. In particular, besides to favor the encapsulation of 3-MCA at the injection site, IFN-γ played an antitumor role enhancing

MHC class I expression on the tumor cell surface and favoring the recognition of tumor antigens by T-cells.

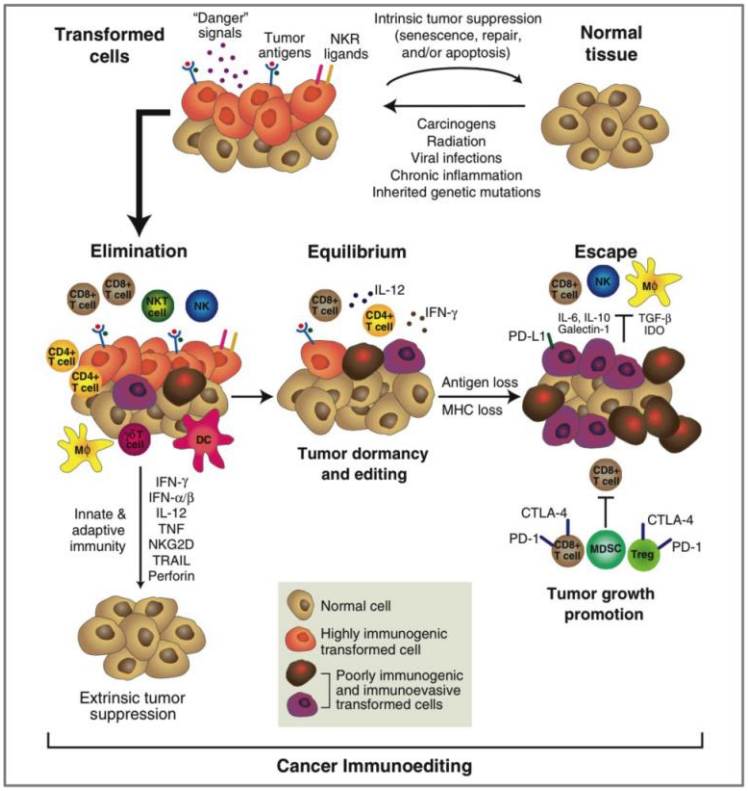

Fig. 3. Cancer immunoediting. Cancer immunoediting is the emerging concept of immunosurveillance and consists of three dynamic phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immunity work together to destroy developing tumors. If cancer cell variants are not destroyed, they may enter the equilibrium phase, in which its outgrowth is prevented by adaptive immunity also for the lifetime of the host. Editing of tumor immunogenicity occurs in the equilibrium phase. However, tumors cells may develop defects in antigen processing or presentation and become insensitive to adaptive immunity. These cells can enter the escape phase and induce an immunosuppressive state within the tumor microenvironment. In this condition, tumor cells cause clinically apparent disease.
From: Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. R. Schreiber et al.; *Science*. 2011.

Furthermore, the two step model of DMBA/TPA, in which the pro-tumorigenic role of the inflammatory component is substantial, also highlights the coexistence of antitumor immunity[24]. For example γδ T-lymphocytes[25] and $CD8^+$ T cells conferred protection differently from $CD4^+$ T cells[26] and Langherans cells[27,28]. In addition, effector molecules and cytokines differently influenced DMBA/TPA-induced papilloma development, as we will discuss later. IL-12 contributed to antitumor effect[22], while IL-23[29] and IL-17 promoted tumor growth[30,31].

Surprisingly, IFN-γ played an opposite role in DMBA/TPA model compared to 3-MCA model[23]. This observation explains that the role of a single immune cell or molecule also depends on the tumor microenvironment.

The equilibrium phase is the "immune-mediated tumor dormancy"[18] in which antitumor immunity contains but does not eliminate residual malignancy. It has been showed that WT mice treated with low doses of 3-MCA displayed small stable masses at the site of MCA injection. The same treatments given with a mixture of monoclonal antibodies that deplete $CD4^+$ and $CD8^+$ cells increased the fibrosarcoma growth compared to NK cell depletion. These data underline the importance of adaptive immunity in tumor dormancy[32].

The escape phase shows the defeats of the antitumor immunity to control tumor growth and derives from changes at the level of both tumor and immune cells. Results demonstrated that neoplastic cells could escape the immunosurveillance through central and peripheral tolerance in which T lymphocytes self-reactive against tumor antigens were eliminated in the thymus or periphery, respectively. Tumor cells also acquired defects in the machinery involved in tumor-antigen processing and presentation. Changes in classical and non classical MHC class I antigens, as well as in the dimeric transporter associated with antigen processing, TAP1 and TAP2, and in β2-microglobulin (associated with the α chain of MHC class I molecules), have been identified in malignant cells. Furthermore, transformed cells lost the ligand for NKG2D limiting NK cell activation and expressed inhibitory ligands of T-cell cytotoxic activity such as B7-1, B7-2, HLA-G and PD-L1[18]. In addition, tumor cells avoided cancer immune-surveillance releasing immunosuppressive cytokines (IL-10, TGF-β, IL-4, IL-13) that attracted tumor-associated macrophages (TAM), discussed below, and $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) that inhibited cytotoxic activity of $CD8^+$ T cells. In particular, it has been showed that TGF-β inhibited dendritic cell (DC) activation, captured aminoacids necessary for T cell function and converted effector T cells in Tregs. Different immune mediators such as cytokines and

chemokines drive the direction of the balance between antitumor (IL-12, IFN-γ) and tumor-promoting inflammation (IL-6, TNF-α, IL-17, IL-23)[18] (Fig. 4).

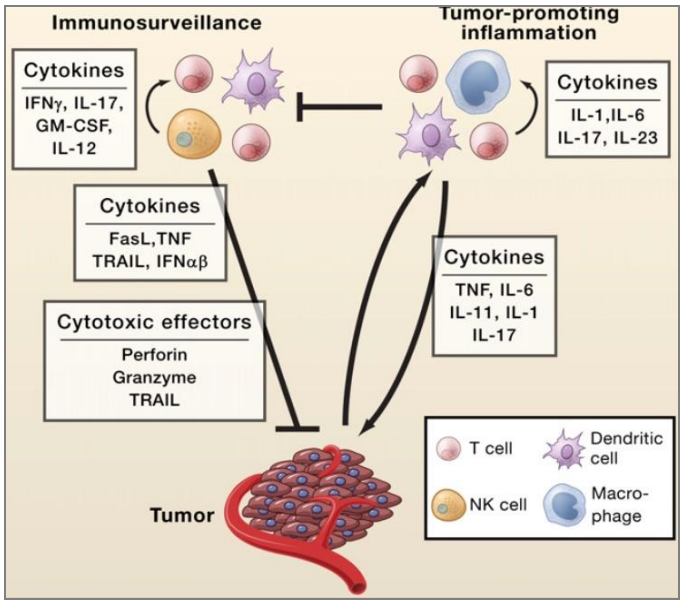

Fig. 4. Balance between Immunosurveillance and Tumor-promoting inflammation. Tumor-promoting cytokines including IL-6, IL-1, IL-23 and IL-17 act on immune and malignant cells to move the balance toward tumor promotion. As a consequence, knowledge of soluble mediator profile for each tumor can be more relevant than tumor-infiltrating lymphocytes.
From: Immunity, inflammation, and cancer. M. Karin; *Cell*. 2010.

As a consequence, knowledge of soluble mediator profile for each tumor can be more relevant than tumor-infiltrating lymphocytes.

This paragraph emphasizes the concept that the immune system does not only defend the host against tumor development but also has the ability to promote tumor growth. These dual effects of the immune system on developing tumors lead to redefine the cancer immunosurveillance hypothesis into the dynamic concept of cancer immunoediting.

1.3 Tumor-associated macrophages (TAMs) and the emergent role of tumor-associated neutrophils (TANs) and myeloid derived suppressor cells (MDSCs) in tumor progression

Macrophages are the most abundant leukocytes in the tumor microenvironment involved in all phases of tumor progression including tissue remodelling, tumor cell growth, angiogenesis, invasion, extravasation and metastasis[33]. Several lines of evidence highlighted the correlation between TAM and poor prognosis such as in Hodgkin's lymphoma, gastric cancer, head and neck squamous carcinoma[34]. Surprisingly, in colorectal cancer (CRC) their role seemed to depend on localization: peritumoral macrophages showed better prognosis compared to intratumoral infiltrate[35].

The bone marrow (BM) is the primary source of monocytes that originate TAMs[36]. Circulating BM-derived $LY6C^+$ murine monocytes were recruited at the tumor site by the chemotactic factor CCL2/MCP-1 (originally described by Bottazzi et al. in 1983). High levels of CCL2 in tumors were associated with bad prognosis and CCL2 depletion reduced TAM number and lung metastasis in a mammary carcinoma[37]. Recently, the splenic red pulp of tumor-bearing mice has been identified as a reservoir of TAMs driven to the tumor through CCR2 signalling[38]. New evidence demonstrated the CSF-1 and STAT-1-dependent proliferative capacity of TAMs in a genetically engineered murine model of breast cancer[39]. Subsequent investigations are necessary to confirm the accumulation of self-maintained TAMs in human cancer.

Macrophages are versatile leukocytes characterized by strong plasticity over-simplified, as discussed by Mantovani, through an M1/M2 nomenclature[40]. M1 macrophages are classically activated by Th1 cytokines such as IFN-γ, TNF-α or microbial stimuli like lipopolysaccharides (LPS). They express inflammatory cytokines such as IL-1β, IL-12, TNF-α, IL-6, IL-23 and chemokines like CXCL9, CXCL10 and produce reactive nitrogen species (RNS) through iNOS. M1 cells play anti-microbial and anti-tumor function and drive Th1 response. In particular, IL-12 favors NK cell and cytotoxic T lymphocyte (CTL) activation[34,35].

In contrast, M2 macrophages are activated by Th2 anti-inflammatory cytokines such as IL-10, IL-4, IL-13, TGF-β and chemokines including CSF-1/M-CSF, CCL5, CCL7 and CCL8. They release immunosuppressive cytokines, such as IL-10, TGF- β, that promote Th2 response. In addition, these cells release anti-inflammatory chemokines like CCL17, CCL18, CCL20, CCL22 and CCL24, which attract Tregs and avoid CTL response. M2 macrophages are poor antigen presenting cells (APC) and display pro-tumor functions. The expression of Ym1, Fizz1, Arginase 1 and scavenging receptors is typical of M2 murine macrophages[34,35] (Fig. 5).

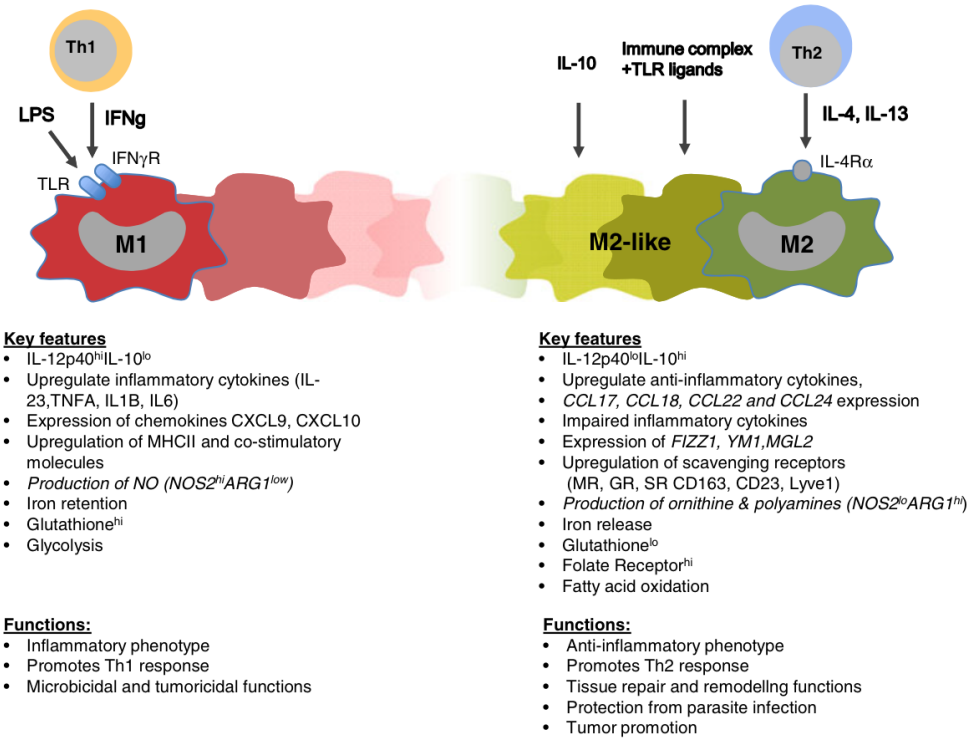

Fig. 5. Spectrum of macrophage polarization. The figure represents a simplified scheme showing macrophage phenotype, with M1 and M2 states representing two extremes of this continuum. Variation among the M2 state is indicated as M2-like phenotype. The most key polarizing stimuli, phenotypic features, and functions are presented for M1 and M2 macrophages. The expression of Ym1, Fizz1, and Mgl2, as well as the NOS versus Arg phenotypes, is specific to M2 polarized mouse macrophages. Similarly, the expression of CCL17, CCL18, CCL22, and CCL24 are found in human M2-polarized macrophages.
From: Tumor-associated macrophages: functional diversity, clinical significance, and open questions. S. Biswas et al.; *Semin Immunophatol*. 2013.

Several data support the M2-like phenotype of TAMs in both human and murine tumors although this cannot be generalized to TAMs in different tumor regions. Van Ginderachter et al. identified at least two TAM subsets on the basis of Ly6C, MHC class II, CX_3CR1 and CCR2

expression in mouse lung carcinoma: $CD11b^{hi}F4/80^{hi}Ly6C^{lo}$ TAM subsets, defined as MHC-II^{lo} and MHC-II^{hi} TAMs. Both subsets derived from the same $Ly6C^{hi}$ tumor-infiltrating monocyte precursor ($LY6C^{hi}CCR2^{hi}CX3CR1^{int}$MHC class II^-), but were located in different oxygenized regions. MHC-II^{lo} TAMs resided in more hypoxic regions and expressed higher levels of M2 markers like arginase and hypoxia-regulated genes than MHC-II^{hi} TAMs. Hypoxia did not alter the number or the polarization of tumor-infiltrating macrophages, but regulated the expression of some M2-like genes such as iNOS, GLUT-1, VEGF and hypoxia responsive genes in MHC-II^{lo} TAMs[41,42]. As a result, the definition of macrophage subsets in different tumors is necessary to define the strategy of treatment.

As mentioned, TAMs influence many aspects of tumor progression such as tumor angiogenesis. When tumors exceed in diameter they need new blood vessels carrying nutrients and oxygen. Hypoxia led HIF-1 α activation which in turn promoted the transcription of genes favoring tumor angiogenesis (such as *VEGF*, *EGF*, *IL-8*, *IL-6*, *TNF-α*, *TGF-β*) in both tumor cells and in resident macrophages[43]. In particular, $Tie2^+$ macrophages (TEMs) had potent angiogenic property and the Tie2 ligand, angiopoietin-2 (Ang-2), favored TEM distribution along blood vessel surface. M-CSF displayed pro-angiogenic functions up-regulating Tie2 on TEM surface[44]. In addition, TEMs favored tumor cell intravasation forming clusters with neoplastic cells around blood vessels. Depletion of $Tie2^+$ TAMs affected angiogenesis and metastasis[45]. In addition, TAMs are rich source of matrix-degrading proteases such as MMP-2, MMP-7, MMP-9, MMP-12, plasmin, urokinase-type plasminogen activator (uPA), which are know to promote pro-angiogenic factor release in the extracellular matrix [43].

Among the protumoral mechanisms, TAMs, as well as tumor cells, expressed non classical MHC class I molecules such as HLA-G, which limited NK cell activation through the binding to NKG2D[33]. In addition, TAMs produced ligands such as PD-L1, PD-L2, B7-1 and B7-2 for inhibitory receptors including PD-1 and CTLA-4 expressed on T-cell surface, favoring thereby

anergy of self-reactive T-cells. More simply, MHC^{low} TAMs displayed weak antigen presenting capacity to T-cells[33].

As described, TAMs directly secreted an array of immunosuppressive cytokines such as TGF-β, and IL-10, which dampened CTL functions and up-regulated Foxp3 in $CD4^+$ T cells, respectively. Moreover, chemokines recruited Tregs showing another indirect immunosuppressive mechanism[33].

As discussed above, M2-like TAMs expressed high level of Arginase 1 which exhausted arginine residues necessary for the membrane expression of CD3 ζ chain, preventing T-cell function[46]. Furthermore, it has been described that TAMs promoted metastasis through the formation of pre-metastatic niches which enhanced homing of circulating tumor cells[47]. In fact, TAM depletion showed reduced lung metastasis in murine breast cancer[48].

This evidence suggests a new therapeutic strategy to target specific TAM subsets in each human tumor (by depletion or repolarization) or their pro-tumorigenic mechanisms, described above[33]. Interesting perspectives derived from the use of the chemotherapeutic agent, Trabectedin, which selectively depleted mononuclear phagocytes, including TAMs, through the activation of caspase-8[49].

Despite copious studies regarding the role of TAMs in the tumor microenvironement, many results have increased the interest on tumor-associated neutrophils (TANs). A recent published meta-analysis concerning 20 articles and 3946 patients have evaluated the prognostic value of TANs in different human cancers. The results of this analysis indicated the association between high density of intratumoral neutrophils and unfavorable survival in head and neck carcinoma, non-small-cell lung cancer and renal carcinoma, but not in gastric cancer. The authors have discriminated among intratumoral, peritumoral and stromal infiltrate, but the low number of papers regarding the last two compartments prevented statistics. In addition, this work showed a better prognostic value of CD66b as human TAN marker than CD15[50].

Fridlender et al. have recently demonstrated that like TAMs, also TANs could feel the influence of the tumor microenvironment and polarize versus N1/N2 neutrophils in a murine model of transgenic lung adenocarcinoma and mesothelioma cell line transplantation. As reported, N1 neutrophils displayed an anti-tumor phenotype with high levels of neutrophil attractant CXCL chemokines and low level of Arginase1. In addition, they promoted $CD8^+$ T cell recruitment releasing chemokines like CCL3, CXCL9 and CXCL10. *In vitro* experiments showed higher capacity of N1 neutrophils to kill neoplastic cells compared to N2 cells.

TGF-β was the main actor for N2 polarization: TGF-β blocking, through the inhibitor SM16, increased N1 neutrophils, and their depletion promoted tumor growth[51].

Furthermore, IFN-β knockout (KO) mice, transplanted with fibrosarcoma or melanoma cells, showed faster tumor growth with more blood vessels than WT mice. Granulocyte depletion reverted the phenotype of IFN-β KO tumors. In fact, IFN-β favored N1 polarization downregulating transcription factors such as STAT-3 and c-myc that regulated pro-angiogenic factors like VEGF, CXCR4, CXCL12 and MMP9 in neutrophils[52].

Recently, Fridlender et al. have studied how tumor microenvironment could influence TAN phenotype during tumor progression in two murine models of tumor cell transplantation. Although they did not find differences in term of neutrophil numbers, their localization switched from periphery to the core of the tumor, between early (7 days post injection) and late (14 days post injection) tumor progression. Moreover, TANs isolated from early tumors displayed higher capacity to kill co-cultured tumor cells than neutrophils from late tumors. Among the mechanisms by which early TANs displayed anti-tumor activity there was the higher production of TNF-α, H_2O_2 (described below) and NO compared to TANs from late tumors. Only depletion of TANs from late stages reduced tumor growth[53]. Thus, like macrophages, TANs display plasticity and play different roles during carcinogenesis depending on the microenvironment.

Sansom et al have found a correlation between neutrophil chemokine receptor CXCR2 and tumor progression. $CXCR2^+$ cells were almost all $Ly6G^+$ cells, and CXCR2 was the main

regulator of their recruitment[54,55]. In particular they documented the resistance of Cxcr2 KO mice to DMBA/TPA-induced skin cancer. Myeloperoxidase $(MPO)^+$ cells were almost absent in Cxcr2-deficient papillomas. Short-term depletion (2,5 weeks) of $Ly6G^+$ cells in established WT papillomas reduced their number and size[56].

The mechanisms by which neutrophils influence tumor progression can be ROS dependent or not[35]. Upon pathogenic insults, neutrophils expressed oxidant-producing enzymes like NADPH, MPO and superoxide dismutase that caused high concentrations of ROS such as O_2^{--}, H_2O_2 and HOCl to resolve the infection. Interestingly, in 1975, Clark and Klebanoff showed the *in vitro* capacity of neutrophils to kill neoplastic cells through ROS activity, membrane-perforating agents and cytokines including TNF-α and IL-1β[57].

However, data from literature attested that high concentrations of ROS can be genotoxic for cells, favoring alterations such as single strand DNA damage and the formation of 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodG). In particular, MPO used H_2O_2 to generate the hypochlorous acid that promoted the formation of an adduct to the DNA, malondialdehyde-deoxyguanosine (M1dG)[58]. It has been estimated that reactive oxygen species are responsible for tumor initiation in one-third of cancers[59].

Proteins released from neutrophil granules can also play a role in tumor initiation. For example, neutrophil elastase, through insulin receptor substrate-1 degradation, dissociated PI3K and activated the PDGFR proliferation pathway[60]. In addition human neutrophils secreted high levels of oncostatin M, a member of IL-6 family, when cocultured with breast cancer cells. TAN-derived oncostatin M promoted VEGF production from neoplastic cells and increased invasion capacity of breast cancer cells[61]. Another *in vitro* study revealed that hepatocyte growth factor (HGF) released from neutrophils promoted invasiveness of cholangiocellular and hepatocellular tumor cells[62].

Both TAMs and TANs are involved in tumor progression releasing pro-angiogenic factors[63]. Neutrophils are the primary source of the potent pro-angiogenic factor VEGF during

physiological conditions such as the menstrual cycle[64]. However, it has been showed that neoplastic cells stimulated TAN recruitment by releasing potent neutrophil CXCL chemokines like CXCL8, CXCL1 (KC), CXCL5 and CXCL6 leading to tumor angiogenesis[57]. Mentzel et al. have shown a positive correlation between the number of neutrophils and microvessel density in high-grade human myxofibrosarcomas[57]. Matrix remodelling by activated TANs was a possible mechanism which released angiogenic factors such as MMP-9/gelatinase B. Coussens et al. have demonstrated that MMP-9-deficient mice displayed reduced keratinocyte hyperproliferation and incidence of skin tumors[65]. Differently from other cells, neutrophils did not release MMP-9 assembled with its inhibitor (TIMP-1), making it quickly available[57]. Activated neutrophils also produced pro-angiogenic chemokines like CXCL8 that contributed to MMP-9 degranulation which in turn favored IL-8 activation[66]. In addition, Ferrara et al. have identified another potent pro-angiogenic factor released by neutrophils: Bv8. CSF-3 was the major positive regulator of Bv8 expression. Treatments with anti-Bv8 antibodies reduced $CD11b^{+}Gr^{+}$ cells, angiogenesis and tumor growth[67].

In addition to release pro-angiogenic factors, neutrophils also favored endothelial cell proliferation through cell to cell contacts involving specific integrins and selectins[63].

Finally, both TANs and endothelium released H_2O_2 which can activate the transcription factor, ETS-1, that regulated the expression of several angiogenic genes like *u-PA*, *MMP-9*[68].

Many data also displayed the metastatic potential of neutrophils for example favoring the release of β2 integrin[69]. In another study, Tazawa et al. have demonstrated that when a poor tumorigenic and metastatic fibrosarcoma cell line (QR-32) was implanted in syngenic mice using a scaffold, which favored neutrophil recruitment, it became highly metastatic. In addition, granulocyte depletion in the early phase significantly supressed these re-acquired metastatic ability of tumor cells[70].

Finally, a recent discovery has linked circulating neutrophil extracellular traps (NET) to clot formation in several murine models of cancer like leukemia, lung and breast cancer. NET

formation caused cancer-associated thrombosis and could be a possible target in the effort to reduce the frequency of thrombosis and death in cancer patients[71] (Fig. 6).

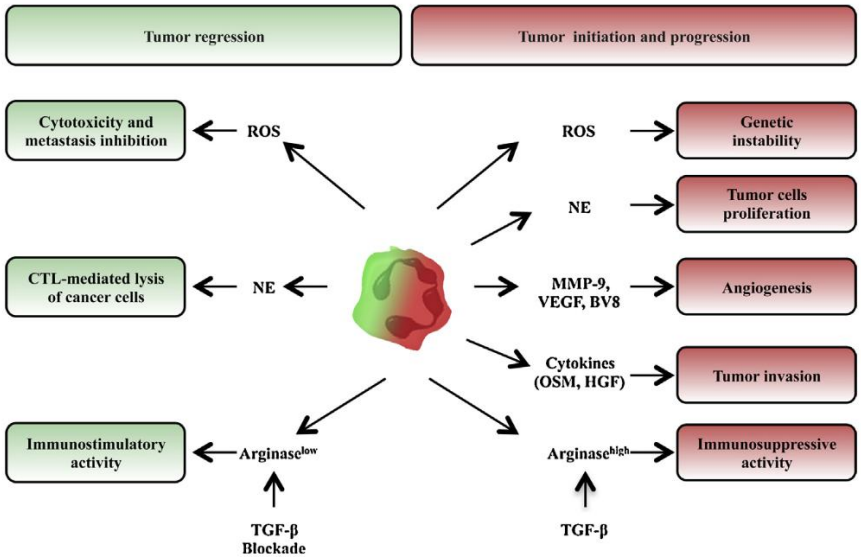

Fig. 6. Emergent role of TANs in tumor growth and progression. TANs may exert both anti-tumoral and pro-tumoral functions. TANs can mediate cancer cell killing through the release of ROS and neutrophil elastase. In contrast, ROS production can favor genetic instability. TANs promote tumor cell proliferation through elastase activity, sustain angiogenesis through VEGF, MMP-9 or Bv8 release, enhance neoplastic cell invasiveness producing soluble mediators (e.g. OSM and HGF) and suppress effective anti-tumoral CD8+ T-cell immunity through the expression of arginase driven by TGF-β.
From: Tumor associated macrophages and neutrophils in cancer. M. R. Galdiero et al.; *Immunobiology*. 2013.

In addition to conditioning macrophages and neutrophils to express an immunosuppressive phenotype, the tumor microenvironment actively recruits and expands an heterogeneous population of immature myeloid cells which are strongly immunosuppressive[72]. Given their characteristics, Gabrilovich et al. suggested to call them "myeloid-derived suppressor cells (MDSC)"[73]. These cells were mobilized from BM by several tumor factors such as CSF-2[74], VEGF[75], IL-1β[76], S100A8 and S100A9[77], which favored their immunosuppressive phenotype. HMGB1 also promoted the recruitment of MDSCs from BM precursor cells and increased MDSC-mediated production of IL-10 favoring an M2 phenotype of TAMs[78].

MDSC composition changes among different tumor histotypes and localizations and depends on tumor-released factors in both mouse and human[79]. Accordingly, as for TAMs and TANs, it is

important not only to attest the presence of these cells, but above all, to characterize MDSC subsets. Initially, observations in tumor-bearing mice identified two markers responsible for MDSC phenotype: CD11b and Gr1[80]. Further characterization discriminated two main subsets: $Ly6C^+$ monocytic MDSCs (MO-MDSCs) and $Ly6G^+$ granulocitic or polymorphonuclear MDSCs (PMN-MDSCs)[81].

Different MDSC subpopulations displayed different mechanisms of immunosuppression. As described for TAMs, the metabolism of specific amino acids like L-arginine by ARG1 or iNOS reduced CD3 ζ chain expression and, as a consequence, T-lymphocyte proliferation[46]. Moreover, IFN-γ-regulated genes such as COX-2, iNOS and gp91phox also played a role in T cell suppression. In particular, COX-2 favored ARG1 induction whereas NADPH oxidase (Nox) subunit (gp91phox) converted oxygen in superoxide anions which induce $CD8^+$ T-cell tolerance[82].

Another mechanism attributed to MDSCs consisted in simultaneous activity of both enzymes, Arg1 and iNOS, which produced ROS and peroxynitrite causing nitration of tyrosines within TCR/CD8 complex, that could not respond to specific tumor-peptide in MHC complex[82].

In addition to these immunosuppressive strategies, it has been described that ADAM17 at the plasma membrane of MDSCs reduced L-selectin expression on the surface of naive T lymphocytes, limiting their recruitment to the lymph nodes[72].

MDSCs expressed Galectin 9, which bound Tim-3 domain on T cells, thereby promoting their apoptosis[83]. Finally, MDSCs decreased the number and inhibited the function of mouse and human NK cells, especially through the interaction with the NK cell receptor NKp30 and through the release of TGF-β1[72]. PMN-MDSCs interfered with T-cell-mediated activity mainly producing ROS, while $F4/80^+$MO-MDSCs acted primarily through iNOS and Arg1, although the distinction was not so clear[79]. Blocking IFN-γ/Stat-1 impaired partially MO-MDSC immunosuppressive function[84].

In humans it is more difficult than in mice to identify the population of MDSCs since the lack of a specific marker such as Gr-1. Recently, at least six human MDSC subsets have been described using a multicolor strategy[85].

Experiments conducted by Corzo et al. have provided the plasticity of MDSCs from the spleen of tumor-bearing mice, to assume a TAM-like phenotype in a condition of HIF-1α-driven hypoxia[86].

However, the relationship between N2 TANs and PMN-MDSCs remains unclear. Recently, Friedlender et al. have developed a trascriptomics analysis to compare mRNA profiles of these cells. This approach showed TAN as a distinct population enriched in chemotactic factors for T-cells but also B-cells, macrophages and neutrophils[87].

Mounting evidence indicates that the tumour microenvironment may influence myeloid cells and convert them to display an immunosuppressive phenotype. Some immunosuppressive mechanisms are common to all myeloid cells, but others are unique to specific populations. Thus, targeting common mechanisms could be a possible therapeutic strategy for certain cancers.

1.4 The role of Complement in tumor development

Complement represents a first line of defence against pathogens or non self-cells (Fig. 7). During carcinogenesis genetic and epigenetic changes can also alter cell membranes and the glycosylation state, becoming an hallmark of cancer[88]. However, there is not experimental evidence supporting the recognition of tumor-associated antigens and further killing of nascent neoplastic cells by the complement system[89]. Indirect assumptions concerning the role of complement in immunosurveillance derived from the presence of several complement proteins in cancer patients. For example increased levels of the anaphylatoxin C3a and C5b-9 (membrane attack complex, MAC)[90] and higher activation of the lectin pathway[91] have been found in patients with ovarian cancer and colorectal cancer, respectively, than control patients. Moreover,

circulating levels of MASP-2 (MBL-associated serine protease-2) from the lectin-pathway have been associated with poor survival in CRC patients[91].

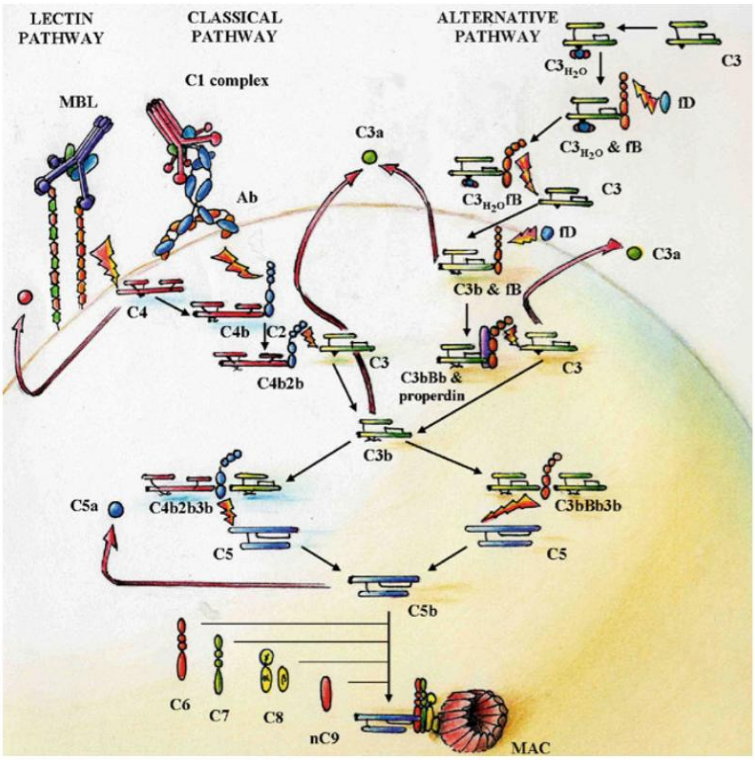

Fig. 7. Cascade of events during the activation of the complement system. Simplified scheme of the pathways of complement activation. Pattern-recognition molecules such as C1q, MBL (mannose-binding lectin), or ficolins bind to surface structures and initiate the formation of the classical C3 convertase. The alternative C3 convertase results from spontaneous hydrolysis of C3 (tick-over). The three complement pathways differ in their mechanisms of target recognition but converge in the activation of the central component C3, which is cleaved by C3 convertase into C3b and C3a. After this activation, C5 is cleaved, and the assembly of the pore-like membrane attack complex (MAC) is initiated. The enzymatic cleavage of C3 and C5 leads to the production of anaphylotoxins C3a and C5a, two important inflammatory mediators and chemoattractants.
From R. Pio et al.; *The Role of Complement in Tumor Growth* in Tumor Microenvironment and Cellular Stress. Springer New York. 2014.

However, neoplastic cells displayed several strategies to influence immunosurveillance such as through the expression of negative complement regulators[88,89,92] (Fig. 8). For example the overexpression of CD55 (C3 and C5 convertase decay-accelerating factors) has been found in prostatic tumors[93]. High levels in urine of the soluble factor H (FH), inhibitor of the alternative pathway, had a negative prognostic potential in bladder cancer[94].

The growing evidence regarding the expression of membrane-bound complement regulator proteins (mCRPs) by neoplastic cells has promoted the use of monoclonal antibodies (mAbs) that recognized tumor antigens to induce complement-dependent cytotoxicity (CDC). In fact, some of these mAbs, such as rituximab, an anti-CD20, have already been used to treat B-cell lymphoma[95].

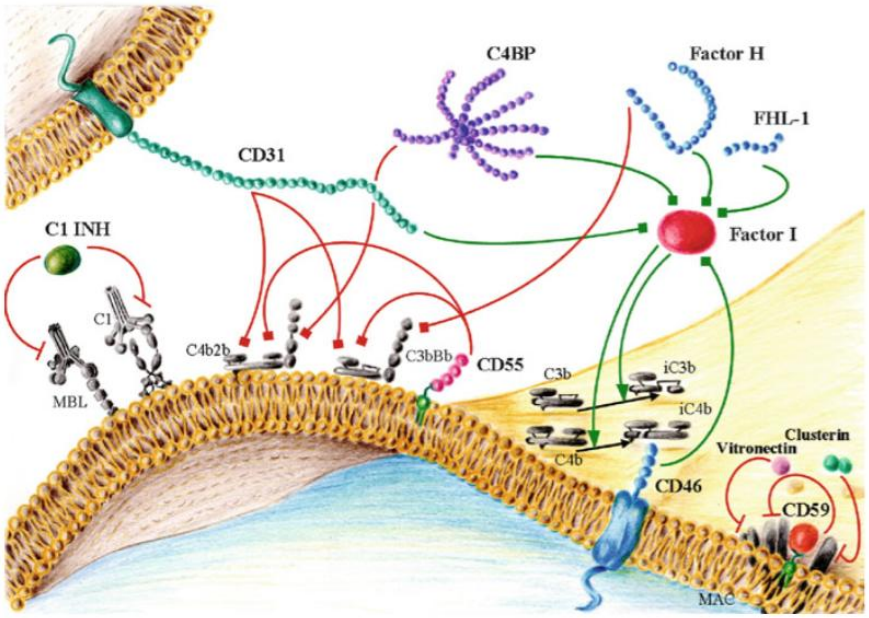

Fig. 8. Main complement inhibitors: soluble proteins and membrane-bound complement regulatory proteins. *Red lines* represent inhibitory activity (when ending in a *bar*) or accelerated decay activity (when ending in a *square*). *Green lines* represent cofactor activity (when ending in a *square*) or protease activity (when ending in an *arrowhead*).
From R. Pio et al.; *The Role of Complement in Tumor Growth* in Tumor Microenvironment and Cellular Stress. Springer New York. 2014.

Among other mechanisms that cancer cells used to avoid complement activation, Moskovich et al. have documented the elimination of MAC through endocytosis process[96]. In addition, membrane sub-lytic levels of MAC activated proliferative and anti-apoptotic pathways[97].

Beyond the CDC mechanism to eliminate nascent tumors, emerging researches have also demonstrated a tumor-promoting role of complement, especially in murine models of cancer (Fig. 9). Since C3 convertase formation and the conversion of C3 in C3b are the point in common for the three pathways of complement activation, the elimination of C3 blocks complement effector functions and allows the study the role of complement in murine models of cancer. Markiewski et al. have shown that after subcutaneous injection with TC-1, a cervical tumor cell line, tumor growth was significantly reduced in the absence of C3[98]. In addition, these authors have investigated the role of the anaphylatoxin C5a, a potent chemotaxin and pro-

inflammatory mediator, in the same tumor model. C5aR deficiency also impaired tumor growth and progression[98]. The same results have been obtained treating 3LL-transplanted tumors with an antagonist of the C5aR[99]. In addition, in a genetic model of ovarian cancer, C3 or C5aR-deficient mice displayed smaller and less vascularized tumors than controls[100].

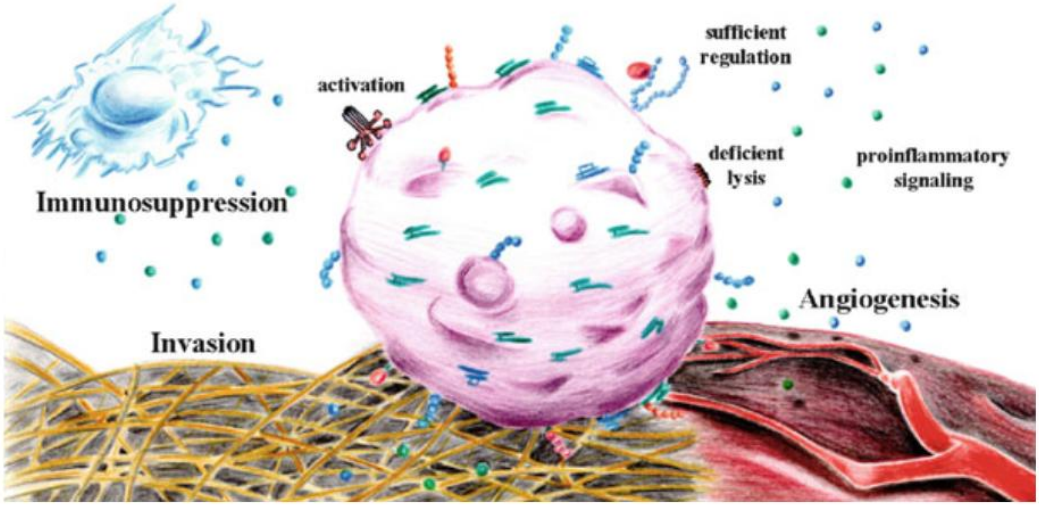

Fig. 9. Potential tumor-promoting roles of complement proteins in the tumor microenvironment.
From R. Pio et al.; *The Role of Complement in Tumor Growth* in Tumor Microenvironment and Cellular Stress. Springer New York. 2014.

An important evidence showed that C5aR blocking significantly reduced MDSCs and immunosuppressive genes (*ARG1, CTLA-4, IL10, PDL1*) [99]. C5a acted as chemoattranctant for MDSCs into tumors[98]. Gunn et al. have found a positive correlation between C5a levels and Treg differentiation in a lymphoma model, suggesting that complement may also regulate Tregs[101]. The interaction between iC3b (fragment of C3b) and complement receptor-3 (CR3) up-regulated the expression of immunosuppressive cytokines (IL-10 and TGF-β) favoring MDSC recruitment[102,103].

It has been described that, under hypoxia, lung cancer cell lines down-regulated mRNA expression of complement regulators like FH, factor I and CD55 favoring complement activation and C3b deposition which could promote cancer progression[104].

Complement may affect tumor progression also playing a role in angiogenesis. There are controversial studies regarding this issue, therefore more investigations are necessary. C5a stimulated human endothelial cell chemotaxis and tube-like formations in vitro[99]. In ovarian cancer, C5a deficiency impaired tumor growth and angiogenesis, suggesting the pro-angiogenic role of complement in tumor progression[88].

Finally complement can also play a role in tumor progression. Tang et al. have demonstrated that C3a reduced the expression of E-cadherin promoting EMT and consequently invasion and metastasis[105]. Moreover, it has been shown that anaphylatoxins C3a and C5a recruited BM-derived mesenchymal stem cells into the tumor, favoring the mesenchymal transition[106]. Complement proteins can also trigger the degradation of the extracellular matrix. Results showed that C5a promoted the release of MMP-9 by C5aR-expressing macrophages[107].

As discussed, the recognition of cancer cells by complement may be an element of immunosurveillance. However, new evidence suggests that when complement fails to protect the host from growing tumor, the balance can move toward tumor-promoting inflammation.

1.5 Important evidence regarding squamous epithelial carcinogenesis: from the role of Mast Cells (MCs) to Circulating Immune Complexes and FcγR activation

In 1999, Coussens et al. have demonstrated the angiogenic potential of mast cells (MCs) in a murine model of SCC in which the early region genes of human papillomavirus type 16 (HPV16) were under control of skin keratin 14 promoter/enhancer. As showed, dysplastic lesions were characterized by angiogenic switch from distal to subepithelial location of dilated blood vessels. MC density increased strongly in the dermal tissue of dysplastic lesions (3-6 months) compared to hyperplastic (1 month) and normal skin. Moreover, angiogenic dysplasia displayed higher activity of MC-specific tryptase/MCP-6 and chymase/MCP4. Tryptase favored fibroblast proliferation in vitro, while chymase acted as angiotensin II-forming enzyme which promoted angiogenesis through the activation of VEGF and MMP-9[108]. Chymase also converted

IL1β, TGF-β and MMP-9 precursors to their active form[109]. MC deficient mice (with the loss of c-Kit) displayed a reduction of skin vascularization and keratinocyte proliferation at 2 months of age. These data support the pro-tumorigenic role of MC during early epithelial carcinogenesis possibly through the regulation of angiogenesis in HPV16 mice[108]. However, several data indicated that MC could switch from the production of immunosuppressive cytokines like IL-4, IL-10, TGF-β, to pro-inflammatory ones, such as IL-6, which converted T-regs into Th17 cells. In addition, the protective role of MCs, in a murine model of CRC, indicated the need to characterize their phenotype in each tumor (tryptase$^+$, chymase$^+$ or both)[109].

De Visser et al. have shown that early lesions of HPV16 mice were characterized by abundant C3 deposition in dermal compartment. However, HPV16/C3$^{-/-}$ mice displayed the same number of MCs and neutrophils (the two most abundant cells infiltrating HPV16 lesions) compared to HPV16 mice at early phases of tumor progression. These data indicated that C3 was not involved in MC and neutrophil recruitment. Moreover, the authors verified the involvement of C3 in MC activation (in term of angiogenic factor release) but the absence of angiogenic variations between HPV16/C3$^{-/-}$ and HPV16 early lesions excluded this contribute.

Dysplastic skins from HPV16 mice displayed an abundant dermal IgG1 deposition that could activate FcγR on inflammatory cell surface, recruiting MCs and neutrophils. As consequence the authors have speculated a complement-independent but antibody-dependent regulation of epithelial squamous carcinogenesis[110].

Subsequent analysis investigated the role of adaptive immunity in the activation of innate immune response in early HPV16 lesions. HPV16/RAG$^{-/-}$ mice, deficient for B and T-cells, showed reduced inflammation in terms of less MC and granulocyte infiltration. In addition, HPV16/RAG$^{-/-}$ lesions displayed reduced levels of VEGF-A, CD31$^+$ cells and epithelial proliferation versus HPV16 skin. B and T cell-deficiency drastically decreased carcinoma incidence. Since CD4$^+$ or CD8$^+$ T cell deficiency did not affect leukocyte infiltration of early lesions, de Visser et al. have hypothesized the pro-tumorigenic role of B cells. However, B-

lymphocytes did not infiltrate skin lesions but released Igs (IgM and IgG1 predominantly), which in turn could activate complement, bind leukocyte receptors and form immune-complexes. HPV16 serum transfer into HPV16/RAG$^{-/-}$ mice restored premalignant characteristics like HPV16 B-cell transfer, indicating the protumorigenic role of B cell soluble factors in the early phases of epithelial squamous carcinogenesis[111].

Andreu et al. have showed that HPV16 deficient mice for B220^{+}CD19^{+} B cells (HPV16/JH$^{-/-}$) displayed a reduced infiltration of MC and Gr1^{+} cells, lower CD31^{+} vessels and keratinocyte proliferation, such as HPV16/RAG$^{-/-}$ mice, suggesting the peculiar role of B cells in premalignant progression. Moreover, HPV16 IgG injection in WT mice was sufficient to induce inflammatory response, demonstrating the premalignant role of antibodies. In addition, the authors have found higher levels of circulating immuno-complexes, CICs, (IgG/C3; IgG/C1q) in HPV16 IgG compared to WT IgG. Since HPV16/FcγR$^{-/-}$ mice showed reduced MC and granulocyte infiltration and angiogenesis, it has been speculated the FcγR-dependent pro-tumorigenic role of CICs in HPV16 mice. CICs may activate FcγR on MC surface promoting angiogenesis and early tumor progression in HPV16 mice (Fig. 10). In addition, they have demonstrated that B cells and FcγR drove M2 polarization of TAMs during squamous carcinogenesis[112].

Because B cells regulate squamous carcinogenesis, and Tnf$^{-/-}$ mice[113], resistant to DMBA/TPA skin cancer, display B cell defects, Schioppa et al. have speculated that B cells were important effector cells for TNF-α–mediated promotion of cancer development. Splenic B-cells, isolated from DMBA/TPA-treated WT mice, injected in Tnf$^{-/-}$ mice, significantly increased papilloma number. In contrast, TNF-α defective B cells failed to restore papilloma development in Tnf$^{-/-}$ mice, suggesting the protumorigenic role of TNF-producing B cells in DMBA/TPA-induced skin cancer[114].

These findings support a model in which activated B cells, CICs and FcγR-activated MCs and neutrophils promote chronic inflammation and favor epithelial squamous carcinogenesis.

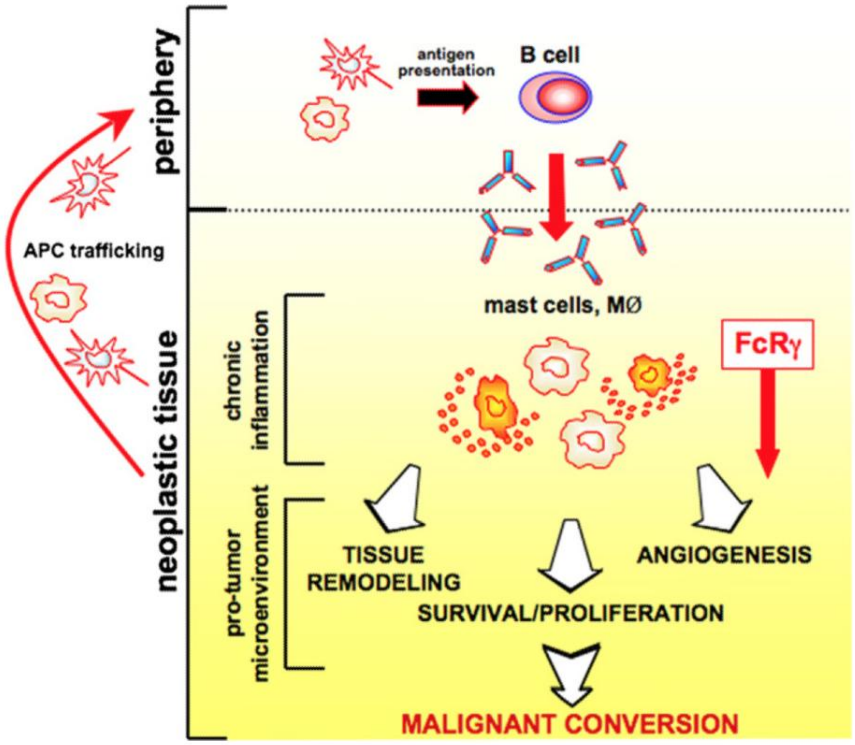

Fig. 10. Activation of the FcγR pathway in SCC. In a genetic model of SCC in which the early region genes of human papillomavirus type 16 (HPV16) is under control of skin keratin 14 promoter/enhancer it has been demonstrated the protumorigenic role of soluble B-cell mediators such immunoglubulins (Igs). Igs accumulate in dermal stroma of neoplastic tissue as vasculature becomes angiogenic, and interact with FcγRs on resident and recruited myeloid cells. This interaction stimulate mast cell and neutrophil bioeffector functions such as tissue remodelling, survival/proliferation, angiogenesis, favoring malignant conversion.
From: FcRgamma activation regulates inflammation-associated squamous carcinogenesis. L. Coussens et al. *Cancer Cell*. 2010.

1.6 Fundamentals of multi-stage DMBA/TPA-induced chemical carcinogenesis and the specific contribution of IL1R-MyD88-NF-κB and IL-23/IL17-pStat3 signalling pathways in skin cancer

The skin represents the first line of defence against pathogens as physical barrier and reservoir of TLRs expressed by keratinocytes and melanocytes. Non-melanoma skin cancer (NMSC) consists of basal cell carcinoma (BCC) and SCC and represents the most frequent cancer in Caucasians, with continuing increase in incidence worldwide. The surgery is the most common treatment to remove clear margins of SCCs. However, in the case of recurring or irregular lesions there is the need of alternative strategies[115].

Differently from transplantation models in which the cancer immunity step has been by-passed, DMBA/TPA-induced skin cancer represents one of the best murine models of multi-step chemical carcinogenesis which studies SCC from the first stages of carcinogenesis, as discussed above. Initiation is an irreversible mechanism, which occurs following a single dose of a carcinogen, the polycyclic aromatic hydrocarbon, DMBA, which mutates *Hras1* gene at the codon 61 (A to G). Mutations in *Ras* gene have been identified in 20% of skin cancers but also in several human epithelial cancers, like pancreatic (90%), colorectal (50%) and lung cancer (25%)[116]. Moreover, repeated treatments with TPA promote the expansion of initiated cells, predominantly keratinocyte stem cells, by activating inflammatory pathways such as EGFR, MAPK, STAT3 and PKC, and stimulating growth factor and ROS release. The two-step skin carcinogenesis is a good model to study human cancer because people are daily exposed to several low doses of both initiators and tumor-promoting agents[117]. The promotion brings to epidermal hyperplasia and, subsequently, papilloma formation (characterized by hyperplastic epidermis with a stromal core), which can also regress. Papilloma growth is expected within 8-20 weeks of progression for the majority of murine strains[117] (Fig. 11).

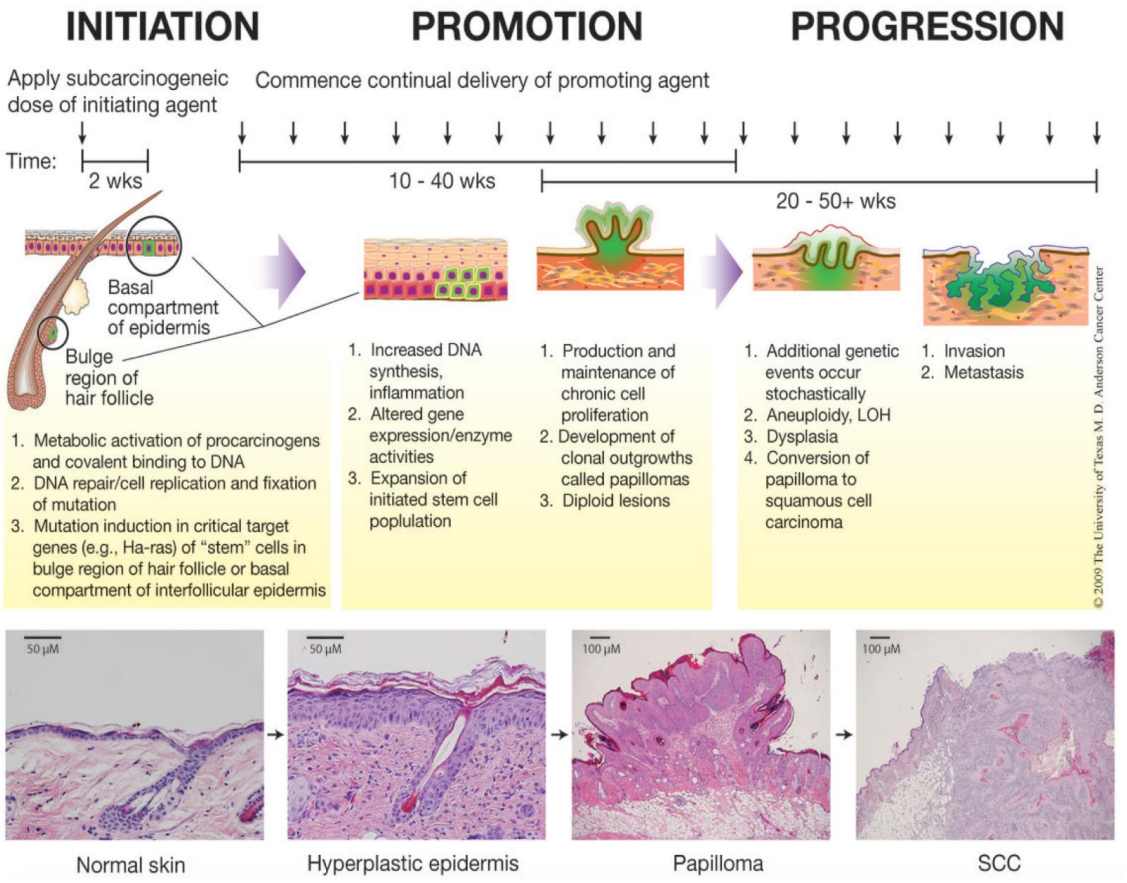

Fig. 11. Two-stage model of skin carcinogenesis in mice. During initiation, topical application of a sub-carcinogenic dose of a mutating agent, DMBA, induces mutations basically keratinocyte stem cells in ras gene. Topical application of a promoting agent, TPA, continues for the duration of the study. However, the number of applications depends on the strain. Papillomas begin to arise after approximately 6–12 weeks of promotion and a fraction begin to convert to SCC after approximately 20 weeks. Representative H&E stained sections of normal skin, hyperplastic skin, a papilloma, and a SCC are presented.
From Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications. E. L. Abel et al.; *Nat. Protoc*. 2011.

It has been described that *p53* gene mutations and trisomy of chromosomes 6 and 7 promote papilloma conversion to SCC. The incidence of carcinoma changes among different strains and depends on genetic background, the dose and the number of TPA treatments[117]. Histologic changes, described above for murine skin carcinogenesis, have been shown to occur concomitantly with modifications in the expression profile of specific keratin proteins. For example, keratin 14 (K14) and keratin 5 (K5) are principally expressed by proliferative basal cells of the epidermis. When keratinocytes differentiate and migrate to the suprabasal layer of the epidermis, they lose the proliferative marker, such as K14 and K5, while the expression of

keratin 1 (K1) and keratin 10 (K10), increases. Loss of K1 and K10 expression, together with high level of K5 and K14, are representative of the progression from murine skin papilloma to SCC and can be detected to verify the grade of the lesion[117].

The use of immune-deficient mice has been instrumental to define the specific contribution of signalling pathways, as well as of immune cells, in the model of DMBA/TPA. Among the transcription factors, which regulate cytokine and chemokine expression, NF-κB represents one the most important factor involved in cancer-related inflammation (CRI), as discussed above. In the skin, the innate immune system orchestrates strongly NF-κB activation. Ligands of TLR/IL-1R superfamily promote the recruitment of MyD88 (except for TLR3), which leads to NF-κB activation[14]. MyD88 is involved in mechanisms of intrinsic and extrinsic CRI. Oncogenic mutations affecting MYD88 have been discovered in human B cell lymphoma[118]. MyD88 contributed to extrinsic mechanisms of CRI in murine model of DEN-induced hepatocarcinoma, 3-MCA-induced sarcoma and $KRAS^{G12D}$-induced pancreatic carcinoma. However, it played a protective role in leukemic virus infections and in AOM/DSS-induced colon carcinogenesis, favoring the IL-18R pathway[119].

Trinchieri et al. have shown that MyD88-deficient mice were protected in DMBA/TPA-induced skin cancer in term of papilloma number and incidence. BM chimeras indicated the contribution of MyD88 from both hematopoietic cells and keratinocytes to promote carcinogenesis. Furthermore, also $Il1r^{-/-}$ mice developed less papillomas compared to WT, even if the papilloma incidence did not change. In addition, tumor growth did not modify after the injection of ras-mutated keratinocytes Il1r KO and WT, respectively. These data indicated that additional factors contribute to the protective phenotype of MyD88 KO mice.

The authors have demonstrated that the keratinocyte expression of pro-tumorigenic factors, such as KC, GM-CSF, TNF, MMP9, and CXCR2 ligands, required the activation of the, IL-1α-mediated, IL1R-MyD88-NF-κB pathway.

MyD88 and Il1r deficiency did not impair keratinocyte proliferation (in term of MAPK activation) but contributed to alter differentiation marker such as K1 and K10.

The authors have explained a mechanism in which RAS-EGFR induction promoted the release of IL-1α by keratinocytes. IL-1α, in turn, activated IL1R-MyD88-NF-κB pathway favoring downstream the production of CXCR2 ligands and the inhibition of keratinocyte differentiation[116]. These data provide the pro-tumorigenic role of IL-1α and MyD88 in murine SCC.

Mittal et al. have analysed the contribution of different TLRs that are upstream of MyD88. They showed that TLR4, but not TLR2 or TLR9, was involved in tumor development. Tlr4 KO mice showed resistance to tumor induction; the number of papillomas and the carcinoma incidence were significantly lower in Tlr4 KO than WT mice. In addition, Tlr4 deficiency was associated with lower levels of inflammatory mediators, like IL-6, IL-17 and IL-1β and granulocyte infiltration. TLR4 expression by both BM and stromal cells was required for carcinoma development in WT mice. Moreover, treatment with ethanol or LPS-binding antibiotic, to inactivate cutaneous bacteria-derived LPS did not change the inflammatory response of WT mice. The authors have identified the endogenous ligand HMGB1, released by necrotic and inflammatory cells, and not skin-colonizing bacteria-derived LPS, as the trigger of TLR4 that was responsible for the recruitment of neutrophils and the generation of a pro-tumorigenic inflammatory response[120].

Despite the role of NF-κB signalling in inflammation associated tumor development, several reports attest the importance of Stat-3 activation to enhance skin tumorigenesis. Stat3 activation, mediated by IL-6, IL-23 and growth factors, promoted the transcription of mitogenic and anti-apoptotic genes such as *cyclin D1* and *bcl-xl* in epithelial cells. Stat-3 favored the transcription of IL-23 while inhibited IL-12 in myeloid cells. IL-23 and IL-12 are members of the same family, produced by phagocytes and DCs. Several data also reported IL-23 production by tumor-associated myeloid cells[121,30]. It has been demonstrated that, although they shared a common

subunit, IL-23 and IL-12 played opposing role in CRI. While IL-12 promoted an anti-tumor IFN-γ-dependent Th1 response, through Stat-1 signalling, IL-23 induced the expansion of Th17 cells through Stat-3 activation. Grivennikov et al. have demonstrated that samples of murine and human CRC displayed high mRNA levels of both IL-23 and IL-17A. IL-23 ablation in the haematopoietic compartment reduced tumor multiplicity, Stat3 phosphorylation, IL-22, IL-6 and IL-17A production in murine CRC. Depletion of intestinal microbiota and TLR/MyD88 inactivation reduced IL-23 expression, Stat3 phosphorylation and IL-17A production. These data underline that genetic mutations can cause epithelial barrier alterations with the entry of bacteria, which activate IL-23/IL-17-mediated adenoma growth in mice[31].

Moreover, Langowski et al. have shown the resistance of Il23 KO mice in the model of DMBA/TPA-induced skin cancer. The authors attributed the protective phenotype of Il23 deficient mice to the higher level of CTLs coincident with lower production of angiogenic factors, such as IL-17 and MMP-9[30]. Despite the tumor-promoting role of IL-23, as a driver of Th-17 cell differentiation and regulator of adaptive immunity, Teng et al. have described that IL-23 antagonized antitumor immune response also blocking NK activity (perforine release and IFNg antitumor effect) through an unknown mechanism, in lung metastasis and 3-MCA-induced fibrosarcoma[29].

Moreover, several papers attest the pro-tumorigenic role of IL-17 in skin cancer. Wang et al. have demonstrated that $Il17^{-/-}$ mice were strongly resistant to DMBA/TPA skin cancer. $CD4^{+}$ T cells are the major source of IL-17. IL-17 deficiency was associated to lower IL-6 production and consequently p-Stat3 activity (regulation of proliferative and anti-apoptotic pathways) with reduced myeloid recruitment in the tumor microenvironment[121]. IL-17 neutralization, in mice with DMBA-induced papilloma, reduced tumor-progression[31].

Based on the knowledge obtained in literature, the two-step model of DMBA/TPA represents the best instrument, which mimics the development of SCC in mice. The use of immune-deficient

mice has been crucial to define the specific contribution of signalling pathways, like IL1R-MyD88-NF-κB and IL-23/IL17-pStat3, as well as of myeloid cells, such as granulocytes in CRI.

2 The long pentraxin PTX3 as a key component of humoral innate immunity and inflammation

2.1 Gene organization, protein structure and expression

Pentraxins are essential components of the innate immunity, which are highly conserved from arachnids to humans. They represent a superfamily of acute phase protein divided in long and short pentraxin on the bases of the primary structure. C-reactive protein (CRP) and serum amyloid P (SAP) components are classic short pentraxins produced by human and murine hepatocytes, respectively. Thereby, the long pentraxin PTX3 is a multimeric glycoprotein of 381 amino acids, which shares the C-terminal pentraxin domain with the short pentraxin and it is coupled with a unique N-terminal region. The primary sequence of PTX3 is highly conserved during evolution with 82% of identical amino acids and 92% of conserved amino acids between human and mouse[122]. However, PTX3 has a unique quaternary structure with eight subunits, assembled together to form an octamer by covalent interactions between cysteines of both N-terminal and C-terminal domain, respectively. Only SAP from *Limus polyphemus* folds into an octamer. It has been described a single N-glycosylation at Asn220 of the C-terminal domain which is involved in the binding to several ligands such as ficolin-1 and P-selectin[123,124] (see below).

The human and murine *PTX3* gene localizes on chromosome 3 and is organized in three exons. The first two exons code for the leader peptide and the N-terminal domain, respectively, while the third exon codes for the C-terminal pentraxin domain. The promoter of both human and murine *PTX3* gene displays potential binding sites for many transcription factors such as Pu1, AP-1, NF-κB, Sp-1 and NF-IL-6[123]. The soluble pattern recognition molecule (PRM) PTX3 has been discovered as a cytokine-inducing gene in fibroblasts and endothelial cells[125]. In fact, while AP-1 regulated *PTX3* basal expression, TNF-α and IL-1β promoted *PTX3* transcription through NF-κB activation. In addition, TNF-stimulation favored PTX3 production through JNK pathway

in alveolar epithelial cells, while high-density lipoproteins (HDLs) activated PI3K/Akt axis in endothelial cells. Moreover, HIF-1α and IL-1R/MyD88 were involved in the up-regulation of *PTX3* gene expression in cardiac ischemia reperfusion in human and mouse, respectively[126]. Furthermore, it has been published recently that TLR4/MyD88 pathway controlled PTX3 production in uroepithelial cells during urinary tract infection (UTI)[127].

PTX3 is expressed by a variety of cell types (e.g. DCs, monocytes, macrophages, alveolar epithelial cells, uroepithelial cells, mammary epithelial cells, smooth muscles cells, endothelial cells, fibroblasts, chondrocytes and adipocytes), except lymphocytes, upon exposure to inflammatory signals like TNF-α, IL-1β, TLR agonists and HDLs, microbial moieties (e.g. LPS, OmpA) or pathogens. Results showed that IFN-γ inhibited the production of PTX3 by DCs, monocytes and macrophages, while IL-10 amplified PTX3 induction by LPS. It has been described that glucocorticoid hormones modulated PTX3 production in a cell-dependent manner, favoring the production in stromal cells while exerting an inhibitory effect in DCs and macrophages[128] (Fig. 12). Moreover, Jaillon et al. have demonstrated that neutrophil granules showed a reservoir of the long pentraxin. Upon microbial recognition and inflammatory signals, PTX3 was released from the granules and localized in NETs. Given the abundance of circulating neutrophils, they represent the major source of PTX3, ready-to-use[129].

The great conservation of both *PTX3* sequence and regulation between human and mouse allows the use of PTX3-deficient mice to address its role especially in host defence and sterile inflammation.

2.2 PTX3 in host defence

The first function reported for PTX3 was the ability to bind a broad spectrum of microorganisms, including *Aspergillus fumigatus* conidia, bacteria such as *Pseudomonas aeruginosa*, *Klebsiella*

pneumoniae and *Uropathogenic Escherichia Coli*[133] (UPEC), viruses like human and murine cytomegalovirus (MCMV) and selected strains of influenza virus[122]. As suggested, PTX3-deficient mice have lead to investigate the in vivo functions of PTX3 in host defence. Interestingly, $Ptx3^{-/-}$ mice displayed increased susceptibility to invasive pulmonary aspergillosis, showing higher mortality than $Ptx3^{+/+}$ mice. PTX3-deficiency was associated with defective recognition of conidia by alveolar macrophages and DC, as well as defective induction of a protective Th1 response[130]. Moreover, treatment with recombinant PTX3 (r-PTX3) reverted the $Ptx3^{-/-}$ mice phenotype in a murine model of aspergillosis[131]. Other studies conducted by Diniz et al. have shown that zymosan recognition, mediated by PTX3, enhanced dectin-1-dependent phagocytosis mediated by macrophages[132]. Moalli et al. have demonstrated that the PTX3 N-terminal domain was responsible for conidia recognition, but the full-length molecule was necessary for opsonic activity. In the presence of C3-deficient serum or anti CR3/CD11b the amplification of phagocytosis, mediated by PTX3, was abrogated. Under these experimental conditions, C3 represented the most potent opsonin, which activated CR3 on neutrophil surface. The presence of PTX3-opsonized conidia enhanced CD11b activation inside the cell, in the phagocytic cup. In addition, PTX3 bound FCγRII/CD32 favoring its activity. FCγRII, in turn, promoted the translocation of CD11b to the phagocytic cap. PTX3-opsonized conidia enhanced neutrophil phagocytic activity through FCγR-dependent CR3 translocation to the phagocytic cap[133]. Other results indicated that PTX3 also displayed therapeutic activity in chronic lung infections by *P. aeruginosa*, the major cause of morbidity in Cystic Fibrosis patients. Mice infected chronically with *P. aeruginosa* and treated with human r-PTX3 presented an enhanced clearance of bacteria in the lungs.

PTX3 recognizes the outer membrane protein (OmpA) of *K.* pneumonia and amplifies the inflammatory response[134]. Recently, Jaillon et al. have demonstrated the protective role of PTX3 in UTI driven by UPEC. PTX3 expressed by leukocytes and uroephitelial cells interacted with

UPEC and promoted the clearance mediated by phagocytes. PTX3-deficient mice displayed an exacerbated UTI. PTX3 represents a good marker of severity of infection suggesting its relevance in the control of infection in humans. In addition, polymorphisms in *PTX3* gene were associated with higher incidence of cystitis and acute pyelonephritis[133].

Several experiments have also highlighted the role of PTX3 in viral infections. For instance, PTX3 bound both human and murine MCMV reducing viral entry in DCs. Consistently $Ptx3^{-/-}$ mice were more susceptible to MCMV infection than $Ptx3^{+/+}$ mice. Human and murine PTX3 also bound influenza virus (H3N2), through sialic acid, and mediated the inhibition of haemagglutination. PTX3-deficient mice were more susceptible than $Ptx3^{+/+}$ mice to H3N2 infection. Since a single amino acid substitution in the haemagglutinin of H3N2 prevented PTX3 binding, it is evident that PTX3 binds selected microorganisms with high specificity[135].

The relevance in humans of the data, which come from animal models of infection, has been demonstrated studying polymorphisms of *PTX3* gene. For example, the homozygous haplotype (h2/h2) in *PTX3* was related with an increased risk of *A. fumigatus* infection as well as with defective expression of PTX3. Functionally, PTX3-deficiency in h2/h2 neutrophils, led to defective phagocytosis and clearance of the fungus[136].

2.3 PTX3 in angiogenesis and matrix remodelling

Angiogenesis is the process, which generates new blood vessels and occurs during physiologic conditions, such as growth, embryonic development and menstrual cycle. However, uncontrolled neovascularization arises during tumor growth. It has been described the existence of pro and anti-angiogenic factors, which controlled the balance of neovascularization. For instance, fibroblast growth factor 2 (FGF2) is a pro-angiogenic factor, which interacts with tyrosine kinase FGFR and heparan sulphate proteoglycans and regulates both endothelial cell proliferation and

chemotaxis *in vitro* and angiogenesis, during tumor progression, *in vivo*[137]. Rusnati et al. have shown that the N-terminal domain of PTX3 contained two binding sites for FGF2. PTX3 prevented FGF2 binding to endothelial cell receptors, leading to specific inhibition of FGF2-induced proliferation and to motogenic activity of endothelial cells *in vitro*. *PTX3* cDNA transduction in FGF2-overexpressing murine aortic endothelial (FGF2-*T*-MAE) cell line inhibited their capacity to generate vascular lesions when transplanted in mice[138,139]. Margheri at al. have demonstrated that the injection of PTX3-transfected breast cancer cells caused a reduction in angiogenesis, compared to non-transfected cells, as a result of FGF2 inhibition[140]. Furthermore, as shown by Presta et al., PTX3 inhibited the mitogenic activity of murine TRAMP-C2 and human prostate cancer cells after stimulation with FGF2, FGF8 or testosterone *in vitro*. In addition, hPTX3 overexpression in TRAMP-C2 cell line, grafted subcutaneously in syngeneic mice, was associated with a reduction of tumor weight, cyclin D1 proliferation and CD31 expression compared to the injection of mock_TRAMP-C2 cell line. These data confirm that hPTX3 also affects the FGF/FGFR angiogenic pathway *in vivo*[141].

It has been demonstrated that the binding of FGF2 to tyrosine kinase receptor favored the proliferation and migration of human coronary artery smooth muscle cells, contributing to the process of restenosis that follows the angioplasty. The PTX3-FGF2 interaction impaired smooth muscle cell proliferation during restenosis. Consistently, a single local endovascular injection of AAV-PTX3 inhibited intimal hyperplasia after arterial injury in rats[142].

Salustri et al. have shown that PTX3 localized within the hyaluronan (HA)-rich matrix formed around the oocyte in the cumulus oophorus complex. PTX3 interacted with TSG-6 and inter-α-trypsin inhibitor (IαI), two molecules required for effective incorporation of the newly synthesized HA into the matrix, an essential step for the stabilization and organization of the matrix of cumuli. As a consequence, $Ptx3^{-/-}$ mice presented a severe deficiency in female fertility because of defective assembly of the cumulus matrix[143].

These results provide strong evidence regarding the involvement of PTX3 in the process of angiogenesis and matrix remodelling. Since neovascularization and extracellular matrix modifications occur during tumor growth, these data also anticipate the involvement of PTX3 in the context of cancer immunology (discussed in the next chapter).

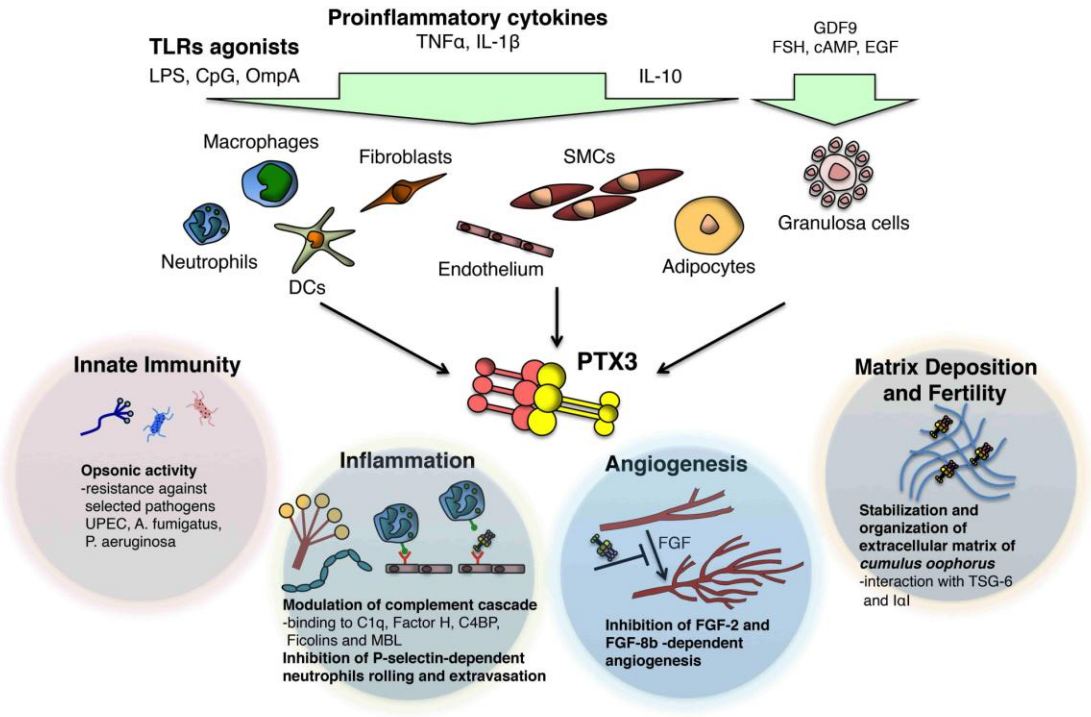

Fig. 12. PTX3: cellular sources and functions. PTX3 is an essential humoral component of innate immunity produced by leukocytes and stromal cells in response to TLR-agonists or pro-inflammatory stimuli such as IL-1β and TNF-α. PTX3 is a functional ancestor of antibodies and acts as opsonin against specific pathogens including UPEC, A. fumigatus and P. aeruginosa. PTX3 is involved in matrix deposition and fertility, favoring HA organization, and in angogenesis, inhibiting FGF-2 and FGF-8b activity. Then, PTX3 regulates inflammation basically modulating complement cascade and P-selectin dependent leukocyte recruitment.
From S. Jaillon et al.; The long pentraxin PTX3 as a key component of humoral innate immunity and a candidate diagnostic for inflammatory diseases. *International Archives of Allergy and Immunology*. 2014.

2.4 PTX3 in inflammation: regulation of complement activation and P-selectin dependent leukocyte recruitment

The first ligand described for PTX3 was the complement component C1q, the main actor of the classical complement pathway activation. The interaction between PTX3 and C1q occurred in calcium independent manner and led to a dual effect on complement activation depending on the way in which the binging occurred. In fact, the interaction between C1q and plastic-immobilized

PTX3 induced the activation of the classical complement cascade and the deposition of C3 and C4. In contrast, when the interaction occurred in fluid phase, PTX3 inhibited the activation of the classical complement cascade blocking functional sites of C1q[144].

In addition, it has been demonstrated PTX3 interaction with three members of the lectin pathway: ficolin-1/M, ficolin-2/L and mannose-binding lectin (MBL). In the case of A. *fumigatus* or *Candida Albicans* infection, PTX3 enhanced the deposition of ficolin-2 or MBL, respectively, on pathogen surface, promoting complement activation[145,146]. PTX3 promoted the binding of Ficolin-1 to late apoptotic or necrotic Jurkat T-cells and favored phagocytosis by human macrophages[147]. These results suggest that during microbial infections PTX3 enhances complement activation favoring the resolution of the disease.

Moreover, Deban et al. have also shown the involvement of PTX3 in the regulation of the alternative pathway. The authors have discovered two binding sites for FH in N-terminal portion and glycosylated pentraxin domain of PTX3. As discussed before, FH is a soluble regulator of the alternative pathway, which inhibits complement activation in different ways such as acting as cofactor of FI, inhibiting the assembly of FB to C3b or promoting C3 convertase decay. PTX3-bound FH remained functionally active[148].

Recently, PTX3 has been showed to interact also with a soluble inhibitor of both classical and lectin pathway, C4b-binding protein (C4BP) in a calcium dependent manner. C4BP inhibits the complement activation acting as cofactor of FI, inactivating C4d, and promoting C3 convertase decay. PTX3-bound C4BP maintained its activity, as described for FH. In addition, when PTX3 bound the extracellular matrix (fibroblast- or endothelial cell-derived) significantly increased the binding of C4BP. PTX3 enhanced the binding of C4BP to apoptotic cell surface limiting complement activation and an exacerbated inflammatory response[149]. In models of sterile inflammation, such as the murine model of myocardial infarction, caused by coronary artery ligation and reperfusion, PTX3 played a protective role limiting an exaggerated inflammatory

response. In fact, Ptx3$^{-/-}$ ischemic lesions displayed higher myocardial damage, neutrophil infiltration and C3 deposition than Ptx3$^{+/+}$, likely through the missing interaction of PTX3 with FH[150].

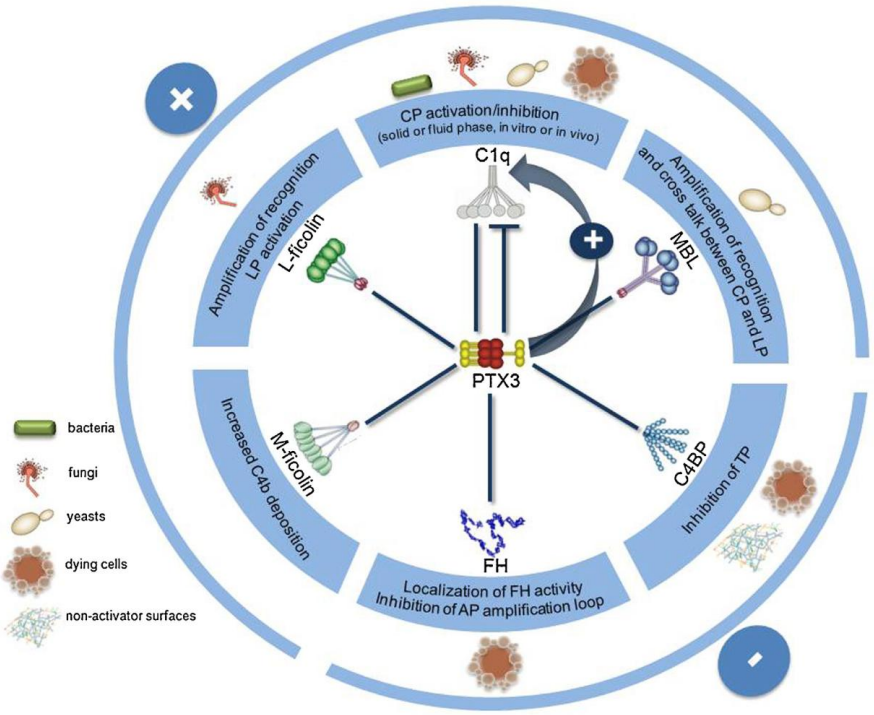

Fig. 13. Roles of PTX3 in the initiation and regulation of the Complement cascade. PTX3 participates in the activation and regulation of Complement cascade through the three pathways, by interacting with C1q, lectins, and with factor H or C4BP. PTX3 plays a complex role in removal of pathogens or dying cells via classical Complement activation. The classical and the lectin pathways are initiated when PTX3 binds to the activation structure (e.g., antigens or microbial moieties). Upon this interaction, downstream Complement components are activated. On the handling of apoptotic cells, the effect of PTX3 on Complement could be divergent depending on the experimental setting (fluid phase or solid phase, in vitro or in vivo). Interestingly, PTX3 interacts with soluble Complement regulators factor H and C4BP, limiting the activation of the terminal pathway and hence Complement mediated excessive inflammation.
From: A. Inforzato et al.; PTX3 as a paradigm for the interaction of pentraxins with the complement system. *Seminar in Immunology*. 2013.

In sterile inflammation, like myocardial stroke, PTX3 avoids an exacerbated complement-mediated inflammatory response, preventing tissue damage (Fig. 13).

Another mechanism through which PTX3 regulates inflammation derived from the interaction with P-selectin. After inflammatory stimuli, P-selectin, expressed by endothelial cells and platelets, binds P-selectin glycoprotein 1 (PSGL-1) on leukocyte surface. This interaction promotes the rolling of leukocytes on endothelium and their migration to the site of

inflammation. Deban et al. have shown that hPTX3 selectively bound P-selectin and not E- or L-selectin, through the glycosidic moiety of the pentraxin domain and competed with the leukocyte PSGL-1 *in vitro*. *In vivo* analysis of leukocyte rolling, after thrombin-mediated vessel stimulation, showed that $Ptx3^{-/-}$ mice displayed higher leukocyte rolling compared to $Ptx3^{+/+}$ mice. Moreover, in a murine model of pleurisy, followed by KC injection, PTX3 administration reduced the accumulation of neutrophils in the pleural cavity.

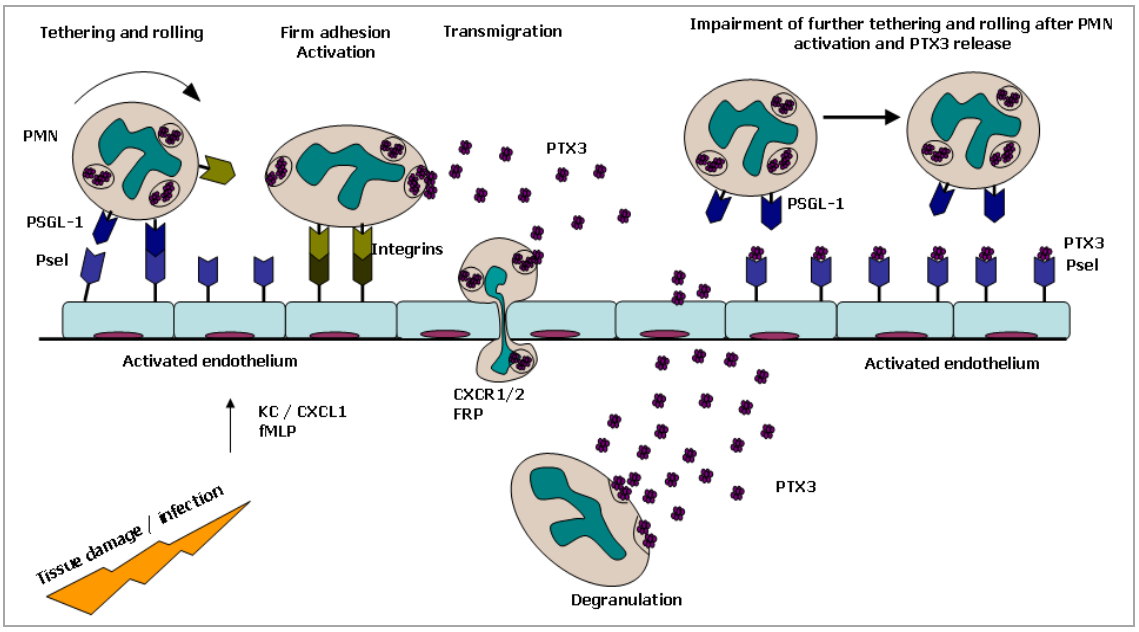

Fig. 14. PTX3 regulates inflammation through the modulation of P-selectin dependent neutrophil recruitment. Interactions of PSGL-1, on neutrophils, with P-selectin, on activated endothelial cells, mediate leukocyte rolling and recruitment at sites of inflammation. Rolling neutrophils release PTX3 from specific granules. It has been demonstrated that PTX3 binds to locally expressed P-selectin, which prevents subsequent interactions with PSGL-1 in murine models of acute lung injury, pleurisy and acute kidney injury. As result, PTX3 avoids an exacerbated inflammatory response limiting neutrophil recruitment to the site of inflammation.
From: L. Deban, et al.; Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat immunol*. 2010.

A similar inhibitory effect has been observed blocking P-selectin. Similar results have been obtained in a murine model of acute lung injury (ALI) and post-ischemic acute kidney injury where the recruitment of neutrophils was a key event to develop the respiratory or renal damage[151,152] (Fig. 14).

All these data indicate a mechanism in which, PTX3, released mainly by neutrophils, acts as negative feedback and prevents an exacerbated neutrophil recruitment blocking the interaction P-selectin-PSGL-1.

Together these results highlight the role of PTX3 in the resolution of microbial infections acting as an opsonin and favoring complement activation. In contrast, PTX3 dampens inflammatory response and tissue damage in sterile condition, limiting complement activation, favoring FH activity, and reducing P-selectin-dependent neutrophil recruitment.

3 Role of PTX3 in cancer

3.1 PTX3 as a tumor biomarker and onco-suppressor gene

A large number of data suggested the role of PTX3 as a marker of inflammation. It has been shown that PTX3 blood levels increased rapidly and dramatically during infectious diseases, including *Mycobacterium tuberculosis* and *Neisseria meningitidis* infections, or vascular diseases such as acute myocardial infarction[153,154,155].

Recently, some papers indicated its relevance as marker of CRI. For example, Planque et al. have validated PTX3 as a novel lung cancer biomarker in the serum of patients (median value of 4,91 ng/ml compared to 1,52 ng/ml of healthy donors)[156]. Moreover, Locatelli et al. have shown PTX3 immunoreactivity in high grade glioblastoma tissues, close to the vessel network and in the extracellular matrix, even if it was absent in many astrocytoma tissues[157]. Recently, data from Gene Expression Omnibus (GEO) have indicated higher expression level of *PTX3* mRNA in bone metastatis of breast cancer patients compared to other metastatic sites[158]. In contrast, data from Oncomine database indicated that *PTX3* mRNA expression is significantly downregulated in several prostatic cancers. Accordingly, Presta et al. have investigated PTX3 expression in 33 prostate adenocarcinoma patients. Immunohistochemistry for PTX3 suggested that the positivity correlated with non-tumor parenchyma or with the extracellular spaces of invasive tumors, around vessels. These data, together with the anti-angiogenic role of PTX3 in murine and human prostatic cancer cell lines, suggested an antineoplastic activity of PTX3 in prostate cancer[141]. Studies in esophageal squamous cell carcinoma (ESCC) have shown that *PTX3* promoter was epigenetically silenced by hypermethylation and PTX3 expression was inhibited compared to adjacent non-tumor tissue. Methylation of the *PTX3* promoter was an early event in tumor development that may be used as biomarker for ESCC diagnosis[159].

Unpublished data from our laboratory indicated that *PTX3* was methylated in selected mesenchymal and epithelial cancers such as leiomyosarcoma, SCC and CRC.

All these data indicate that PTX3, in addition to act as a biomarker of CRI in specific tumors, can be express possibly by infiltrating leukocytes or vessels in the tumor microenvironment. However, PTX3 is silenced in selected mesenchymal and epithelial tumors acting as an extrinsic oncosuppressor gene (see below).

3.2 PTX3-deficiency is associated with increased tumour growth in a murine model of fibrosarcoma chemically induced by 3-methylcolantrene (3-MCA)

Unpublished experiments from our group demonstrated the protective role of PTX3 in a murine model of 3-MCA-induced fibrosarcoma. These experiments showed that PTX3-deficiency was associated to increased fibrosarcoma incidence (Fig. 15).

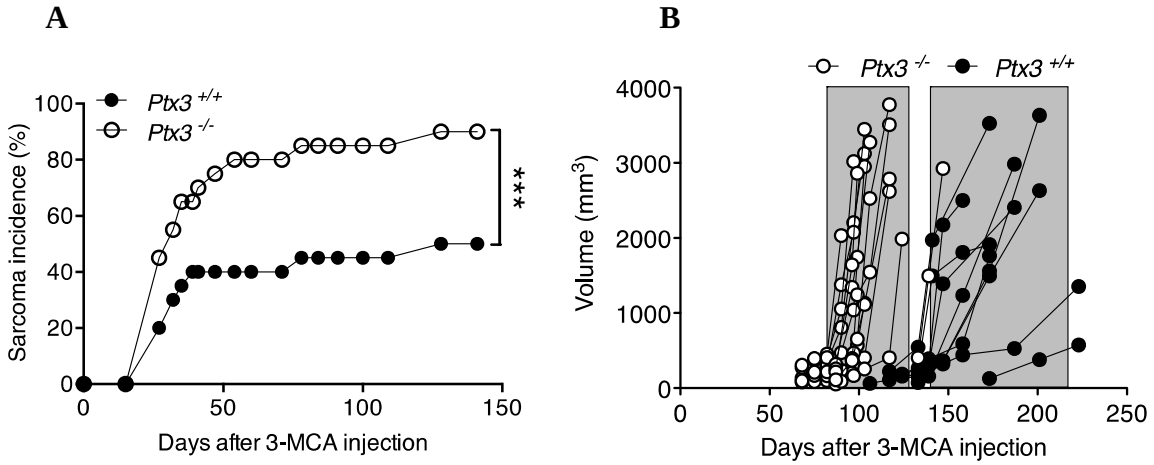

Fig. 15 - PTX3-deficiency increases susceptibility to carcinogenesis.
Incidence (A) and tumor volume (B) of 3-MCA-induced sarcoma in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice. In B, the volume of each tumor is shown. One experiment with 20 mice/group out of 10 performed with similar results is shown. ***: $p<0.001$, paired Student's t test. Adapted from Bonavita, Gentile et. al., in press. Cell.

Moreover, $Ptx3^{-/-}$ sarcomas grew faster and reached the end point earlier than control mice. Rescue experiments performed by administering rPTX3, throughout the duration of the experiment, significantly reduced the tumor incidence, macrophage infiltration, angiogenesis and C3 deposition of $Ptx3^{-/-}$ mice.

During early carcinogenesis macrophages were the primary source of PTX3 in both 3-MCA-injection site and liver. Lower circulating PTX3 levels in $Il1r1^{-/-}$ mice compared to $Ptx3^{+/+}$ mice indicated the involvement of IL-1 in PTX3 production. In 3-MCA tumors, PTX3 expression was low and associated to vessels, infiltrating macrophages and neutrophils, whereas tumor cells expressed variable levels of PTX3. Studies with chimeric mice demonstrated that there were no differences in term of fibrosarcoma incidence between $Ptx3^{+/+}$ receiving $Ptx3^{-/-}$ BM and vice versa, suggesting the importance of both hematopoietic and non-hematopoietic cells as source of PTX3 to give protection.

The biochemical and histological analysis of the injection site showed the association between PTX3-deficiency and increased inflammation in terms of higher leukocyte recruitment at the injection site and pro-inflammatory mediators such as CCL2 and CXCL2. Higher macrophage and monocyte infiltration and angiogenesis ($CD31^{+}$ expression) were observed in $Ptx3^{-/-}$ than $Ptx3^{+/+}$ tumors (Fig. 16).

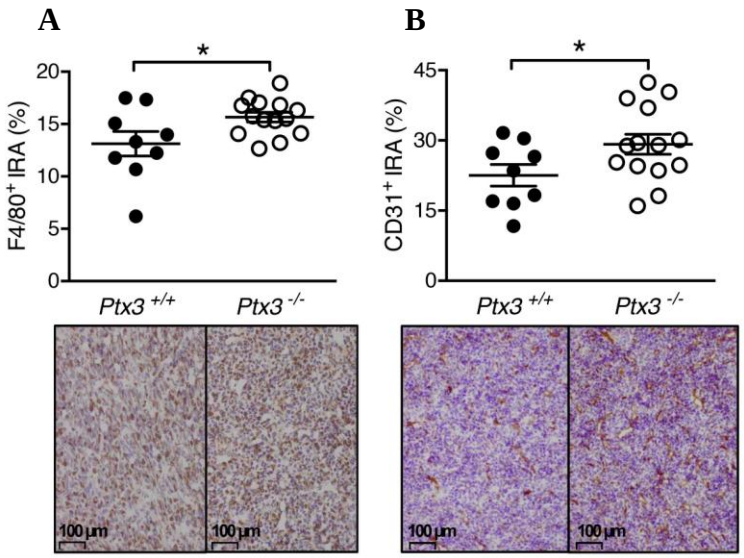

Fig. 16. (A) Analysis of macrophages (F4/80^{+} immunoreactive area, IRA) and (B) angiogenesis (CD31^{+} IRA) in 3-MCA-sarcoma by immunohistochemistry (each dot represents the average of ten fields, mean ± SEM). *: $p<0.05$, unpaired Student's t test. Adapted from Bonavita, Gentile et. al., in press. Cell.

Confocal microscopy displayed that PTX3-deficiency was associated with increased complement activation, in terms of higher C3 and lower FH deposition (Fig. 17A-C). In Ptx3$^{+/+}$ sarcomas, PTX3 and C3 were inversely correlated, whereas PTX3 and FH showed a positive correlation.

In the effort to assess the pathogenic role of increased complement activation associated to PTX3-deficiency, the susceptibility to 3-MCA of C3-deficient and PTX3/C3-double deficient mice was evaluated. The deficiency of C3 reduced sarcoma incidence of Ptx3$^{-/-}$ mice to the level of Ptx3$^{+/+}$ mice (Fig. 17D) and abolished macrophage accumulation of Ptx3$^{-/-}$ tumors.

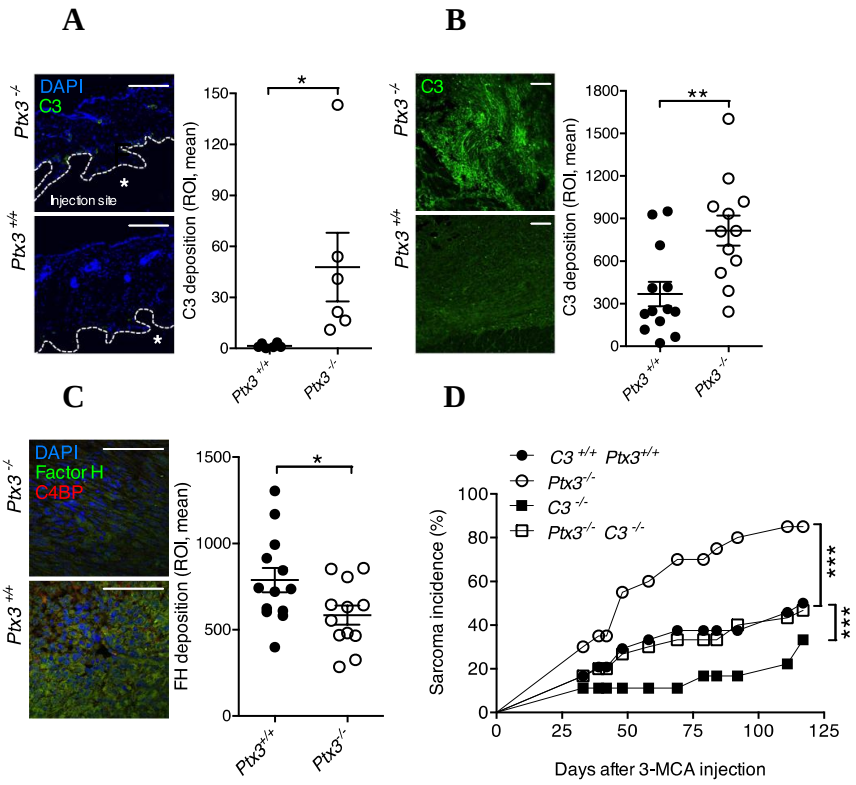

Fig. 17. PTX3-deficiency is associated to increased Complement deposition in 3-MCA-sarcoma.
(A, B): Analysis by confocal microscopy of C3 deposition in the injection site (*) at 7 days (A) or in tumors (B) of Ptx3$^{-/-}$ and Ptx3$^{+/+}$ mice. (C) Analysis by confocal microscopy of Factor H in 3-MCA-tumors. *: p<0.05, **: p<0.01, unpaired Student's t test. Each dot represents the average of mean fluorescence intensity of ten regions of interest (ROI) per tumor. Scale bars represent 150µm. (D) Incidence of 3-MCA-sarcoma in C3$^{-/-}$ and/or Ptx3$^{-/-}$ and Ptx3$^{+/+}$ mice (n=10-15). ***: p<0.001, paired Student's t test. Adapted from Bonavita, Gentile et. al., in press. Cell.

In addition, treatment with a C5aR antagonist (PMX53), throughout the duration of the experiment, rescued the phenotype of Ptx3$^{-/-}$ mice. These data indicated that PTX3-deficiency led to complement activation, in term of C3 and C5a activation and it caused increased susceptibility to 3-MCA.

In vitro assay suggested that C3 deposition was higher in Ptx3$^{-/-}$ than Ptx3$^{+/+}$ fibrosarcoma cells. Moreover, rPTX3 reduced C3 levels on Ptx3$^{-/-}$ cells. In addition, a FH inhibitor, which inhibited the binding of PTX3 to the FH, increased C3 deposition on Ptx3$^{+/+}$ cells whereas did not further influence C3 deposition on Ptx3$^{-/-}$ cells. Interestingly, FH inhibitor abolished the effect of rPTX3 on Ptx3$^{-/-}$ cells, favoring C3 increase. All together these results demonstrated that complement is

an essential component of tumor-promoting inflammation and that the lack of PTX3-mediated FH recruitment is responsible for the higher complement activation of Ptx3$^{-/-}$ tumors.

PTX3-deficiency was associated with increased macrophage infiltration and higher levels of the monocyte attracting chemokine CCL2. Complement activation and C5a in particular favor chemokine production. It was therefore important to assess the relevance of CCL2 in the enhanced susceptibility to carcinogenesis exhibited by PTX3-deficient mice. CCL2 blocking for the duration of carcinogenesis reverted fibrosarcoma incidence of Ptx3$^{-/-}$ mice suggesting that the treatment was effective in condition of exacerbated CCL2 production (Fig. 18).

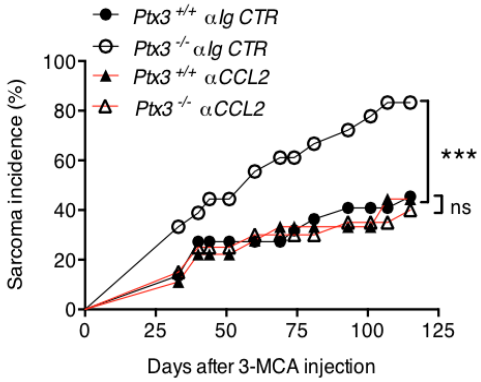

Fig. 18. Role of CCL2 in carcinogenesis and TAM polarization in PTX3-deficient mice. Incidence of 3-MCA-sarcoma in *Ptx3*$^{-/-}$ and *Ptx3*$^{+/+}$ mice treated with anti-CCL2, or irrelevant mAb (n=9-11) (100µg/mouse three times/week) for the duration of the experiment. ***: p<0.001, paired Student's t test.
Adapted from Bonavita, Gentile et. al., in press. Cell.

Since CCL2 has been shown to skew macrophage polarization in an M2-like direction the phenotype of TAM was characterized. Both MHC II ^{high} and MHC II ^{low} TAMs from Ptx3$^{-/-}$ mice showed an M2-like phenotype and high level of iNOS.

CRI is potentially a cause of gene instability. Accordingly it was addressed whether the increased tumor incidence observed in Ptx3$^{-/-}$ mice was associated to increased 3-MCA induced gene mutation. Ptx3$^{-/-}$ tumors were associated to increased p53 mutations, compared to Ptx3$^{+/+}$

tumors. In addition, PTX3-deficiency correlated with higher expression of 3-MCA extra-mutations such as 8-OH-deoxiguanosine (8-OH-dG), ROS/RNS-associated DNA damage, or DDR markers such as 53BPI or γH2Ax, compared to $Ptx3^{+/+}$ tissues in early carcinogenesis. Moreover, treatments with aminoguanidine hemisulphate (AG), an irreversible inhibitor of iNOS, in 3-MCA injected mice, reduced the fibrosarcoma incidence of $Ptx3^{-/-}$ mice but also the expression of DDR or ROS/RNS markers. These results suggest that increased CRI in PTX3-deficient mice is associated with increased genetic instability as assessed by the frequency of *Trp53* mutations, oxidative DNA damage and expression of DDR markers.

These results demonstrated for the first time that the regulatory component of the humoral arm of innate immunity, PTX3, acts as an extrinsic oncosuppressor gene in mice in a model of chemically induced mesenchymal tumor. In addition the epigenetic studies discussed above demonstrate that *PTX3* gene is silenced by hypermethylation in selected human tumors.

Adapted from Bonavita, Gentile et. al., in press. Cell.

Aim

Since previous studies by our group demonstrated the protective role of PTX3 in *cancer-related inflammation* (CRI) in a murine model of chemically induced fibrosarcoma, a tumor with mesenchymal origin, we also addressed the role of PTX3 in a murine model of epithelial origin, DMBA/TPA chemically induced skin cancer.

Beyond the effect exerted on macrophages and complement components, we investigated other potential PTX3-related mechanisms in dampening tumor-promoting inflammation in mouse skin carcinogenesis.

4 Results

4.1 PTX3 is produced during skin carcinogenesis

DMBA is a carcinogenic compound acting as initiator in a single cutaneous application, while TPA is a promoter, applied three times weekly during all the period of carcinogenesis. We first analysed the production of PTX3 during DMBA/TPA-induced skin carcinogenesis. As shown in Fig. 19A, PTX3 was strongly produced in the skin 12h after a single DMBA-TPA treatment.

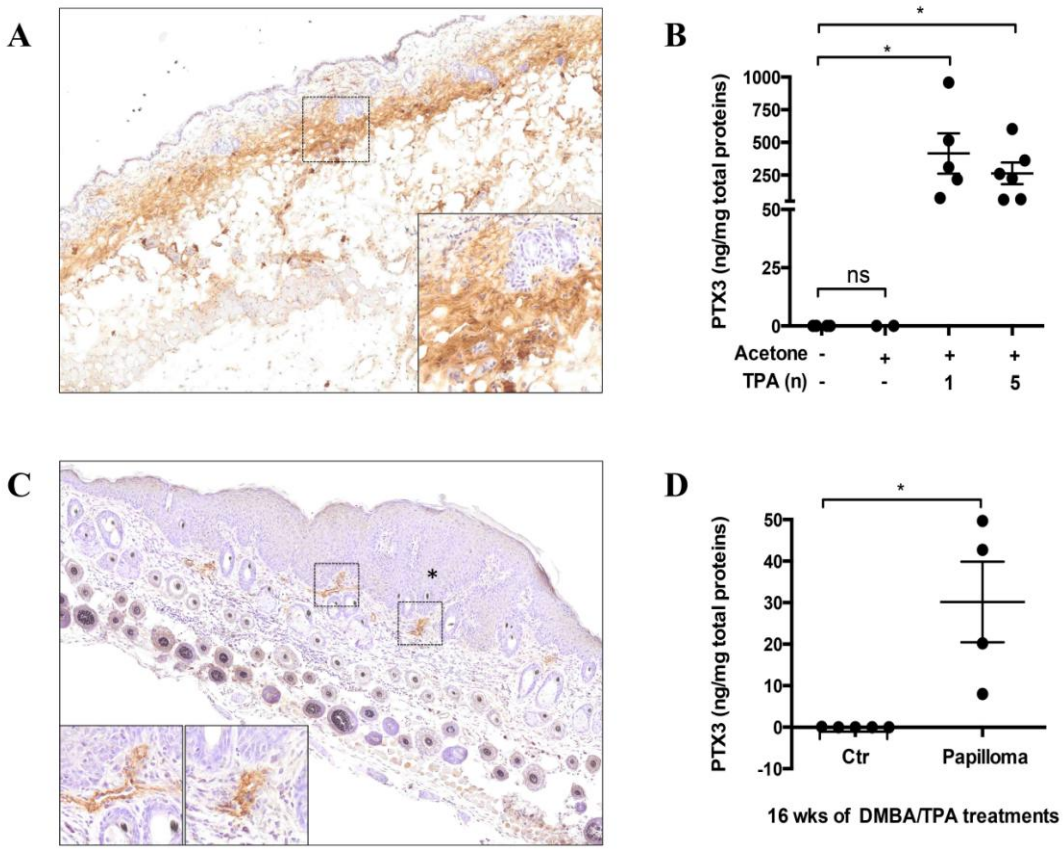

Fig. 19. DMBA/TPA treatment induces PTX3 expression in both early and late carcinogenesis.
(A) Immunohistochemical analysis for PTX3 on $Ptx3^{+/+}$ skins collected 12 hours after the first TPA. One representative photograph of 6 skin sections is shown. (B) PTX3 dosage by ELISA of skin homogenates recovered 12 hours after 1 or 5 TPA. *: $p<0.05$, unpaired Student's t test. (C) Immunohistochemical analysis for PTX3 on $Ptx3^{+/+}$ papillomas recovered 24h after 16 weeks of DMBA/TPA treatments. (D) PTX3 dosage by ELISA of homogenized $Ptx3^{+/+}$ papillomas. *: $p<0.05$, unpaired Student's t test.

ELISA confirmed significantly higher levels of PTX3, 12h after 1 or 5 TPA treatments, following DMBA application, in immunocompetent skins (Fig. 19B). In papillomas recovered 24h after 16 weeks of DMBA/TPA treatments, PTX3 immunostaining was associated with the extracellular matrix (Fig. 19C) and PTX3 levels were higher than in control skins (Fig. 19D). Moreover, confocal microscopy of the skin revealed PTX3 association with infiltrating macrophages (Fig. 20A) and neutrophils (Fig. 20B and 20C) 8h after a single DMBA-TPA treatment and with lymphatic vessels 24h after 16 weeks of DMBA/TPA treatments (Fig. 20D).

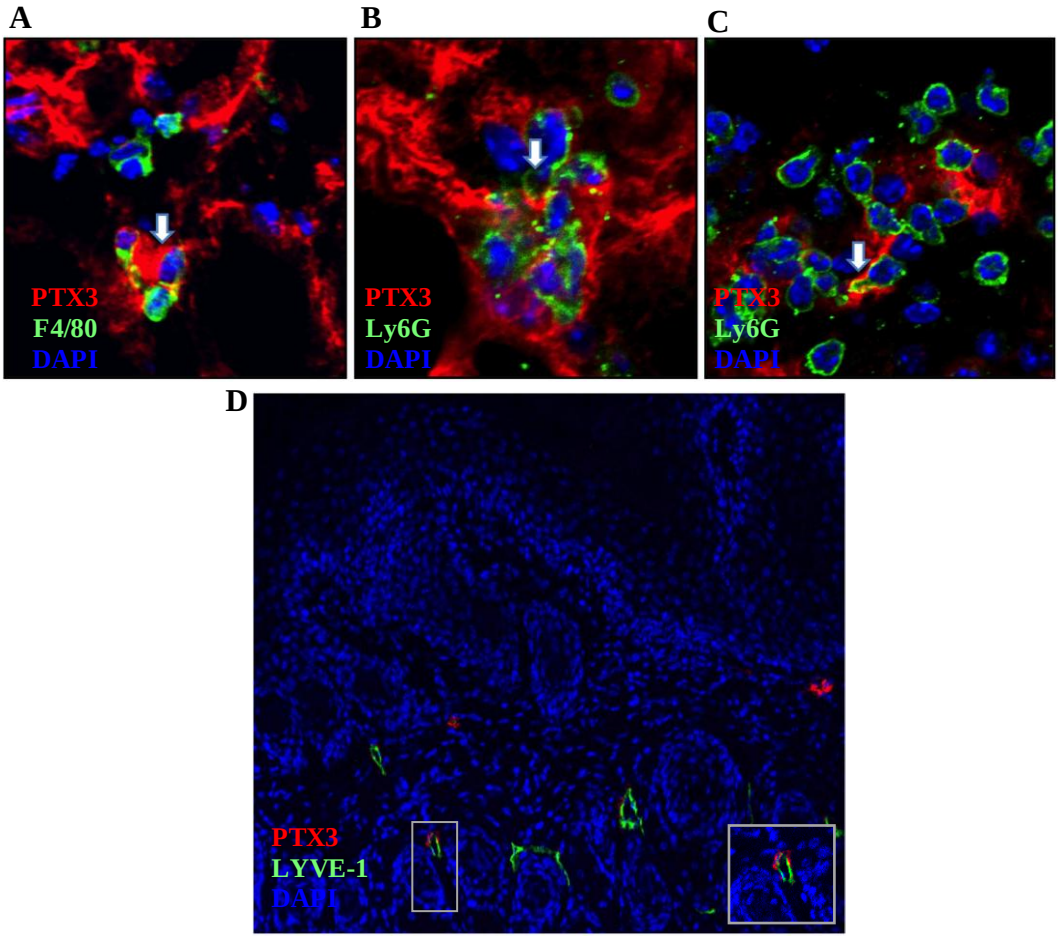

Fig. 20. PTX3 immunostaining is associated to infiltrating macrophages and neutrophils, lymphatic vessels and interstitial stroma. (A-C) Immunofluorescence analysis for PTX3 and F4/80 (macrophage marker) or for PTX3 and Ly6G (neutrophil marker) on skin samples, collected 8 hours after the first TPA. One representative figure shows the production of PTX3 (red) by infiltrating macrophages or neutrophils (green). (D) Immunofluorescence analysis for PTX3 and LYVE-1 (lymphatic marker) on $Ptx3^{+/+}$ papillomas collected after 16 weeks of DMBA/TPA. One representative image displays the presence of PTX3 (red) in the extracellular matrix, and PTX3 association with lymphatic vessels (green).

Collectively, these results demonstrate that PTX3 is abundantly produced in early carcinogenesis by infiltrating leukocytes, vessels and interstitial stroma. Moreover, PTX3 is present in the extracellular matrix also in late carcinogenesis following chronic treatments with TPA.

4.2 PTX3-deficiency is associated with increased incidence, mean number of papilloma and more aggressive lesions

$Ptx3^{-/-}$ and $Ptx3^{+/+}$ mice on C57BL/6J background were compared for susceptibility to 25 μg DMBA initiation followed by 4 μg TPA, three times weekly, for 30 weeks of promotion. As shown in Fig. 21A, nearly 100% of $Ptx3^{-/-}$ mice developed at least one papilloma in comparison to $Ptx3^{+/+}$ mice with less than 80% of papilloma incidence (p=0.0004). Moreover, PTX3-deficient mice were more susceptible to DMBA/TPA-induced skin carcinogenesis than $Ptx3^{+/+}$ controls also in term of papilloma number (p=0.0005)(Fig. 21B).

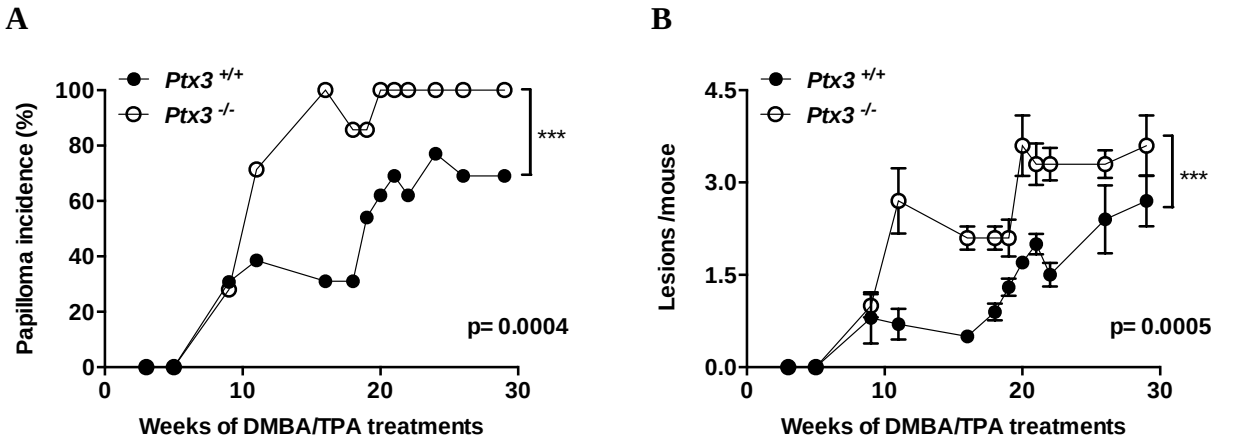

Fig. 21. PTX3 plays a protective role in DMBA/TPA-induced skin papillomas. (A) Percentage of papilloma incidence and (B) number of papillomas per mouse in each group (mean ± SEM). Groups of female $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice are treated with 25 μg DMBA, and 1 week later, three times weekly with 4 μg TPA for 30 weeks as described in Materials and Methods. Mice are monitored for skin papilloma development for 30 weeks. One experiment with 10 mice/group of 4 performed with similar results is shown. ***: p<0.001, paired Student's t test. Adapted from Bonavita, Gentile et. al., in press. Cell.

In addition, histological classification resulting by hematoxylin and eosin staining and certain macroscopic characteristics, such as tumors growing downward as well as tumors attached to the underlying muscle layer, showed higher carcinoma incidence of $Ptx3^{-/-}$ lesions compared to competent mice (p=0.009) (Fig. 22A-D). In DMBA/TPA-induced skin carcinogenesis, histological changes occur concomitantly with modifications in the expression profile of specific keratin proteins. Keratinocytes differentiate and migrate to the suprabasal layer of the epidermis losing the proliferative marker K14 and acquiring the differentiation marker K1.

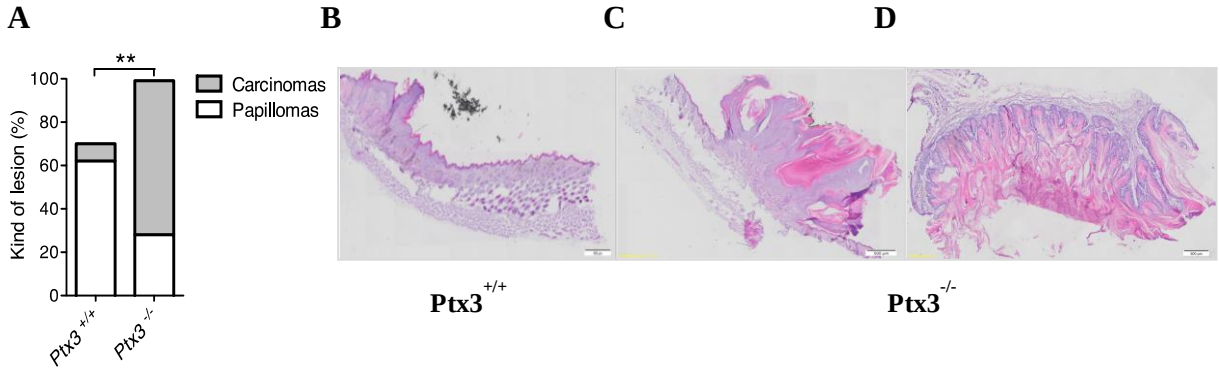

Fig. 22. $Ptx3^{-/-}$ mice display higher carcinoma incidence compared to $Ptx3^{+/+}$ mice. (A) Histological classification of lesions developed in PTX3-deficient and competent mice after 30 weeks of DMBA/TPA. ***: p<0.01, Chi-square test. (C, D) Two representative sections of $Ptx3^{-/-}$ lesions. (D) One representative section of $Ptx3^{+/+}$ lesions stained with hematoxylin and eosin.

Loss of K1, together with high level of K14, are representative of the progression from murine skin papilloma to SCC and can be detected to verify the grade of the lesion[117]. Analysis of K1 and K14 expression by confocal microscopy showed higher K14 expression level and lower K1 intensity of $Ptx3^{-/-}$ compared to $Ptx3^{+/+}$ lesions (Fig. 23A-D). All these data indicate that PTX3 is involved in controlling the incidence and number of papillomas and affects malignant progression from papilloma to SCC. Previous results showed the higher tumor susceptibility of $Ptx3^{-/-}$ mice in a model of 3-MCA-induced fibrosarcoma. All these data suggest the protective role of PTX3 in both epithelial and mesenchymal tumors.

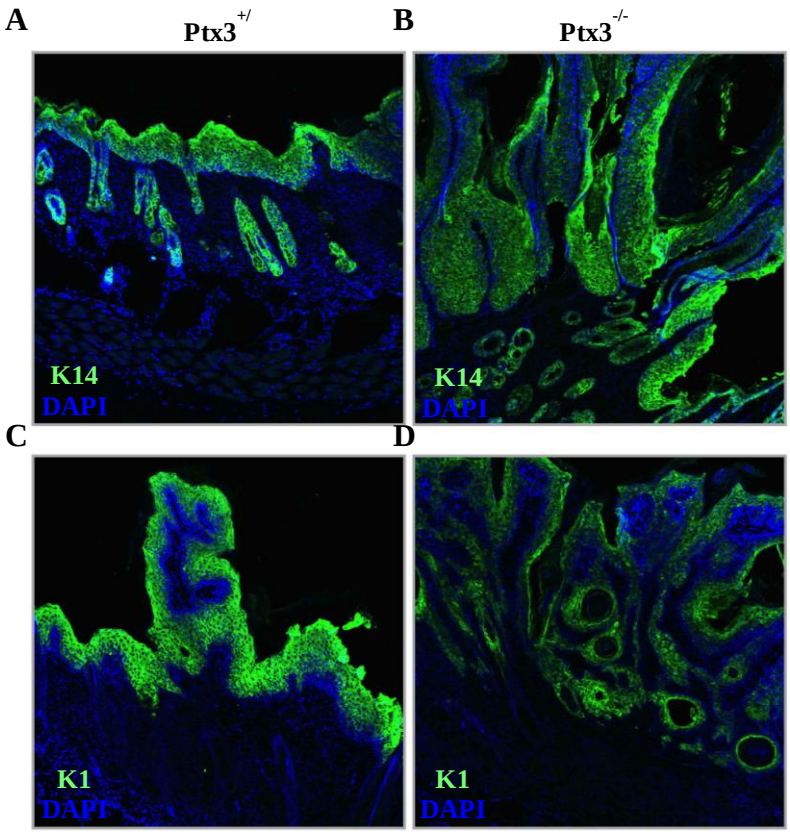

Fig. 23. Expression profile of specific keratin proteins. Confocal microscopy for K14 (proliferative marker), K1 (differentiation marker) and DAPI (nuclear marker) on PTX3-deficient and competent papillomas collected after 30 weeks of DMBA/TPA. (A, B) Two representative images showing cutaneous K14 expression. (C, D) Two representative images showing cutaneous K1 expression in both $Ptx3^{+/+}$ and $Ptx3^{-/-}$ lesions, respectively.

4.3 PTX3-deficiency is associated with higher inflammation in early carcinogenesis

The following experiments had the aim to explain the molecular mechanisms, which underlay the protective role of PTX3 in mouse skin carcinogenesis. The long pentraxin PTX3 is an essential component of the humoral arm of innate immunity, which regulates inflammation basically through three mechanisms. PTX3 activates and regulates the complement cascade, controls leukocyte recruitment in a P-selectin-dependent manner and interacts with FcγR miming antibody properties[128]. Thus, we characterized CRI in $Ptx3^{-/-}$ mice. As shown in Fig. 24A, after 3 weeks of DMBA/TPA treatments, PTX3-deficient skins were associated with increased neutrophil infiltration compared to PTX3-competent skins. Accordingly, the MPO content (Fig. 24B) was significantly higher in skin homogenates from $Ptx3^{-/-}$ than $Ptx3^{+/+}$ mice. In addition,

CD31 immunostaining showed expanded angiogenesis in Ptx3$^{-/-}$ compared to Ptx3$^{+/+}$ skins (Fig. 24C).

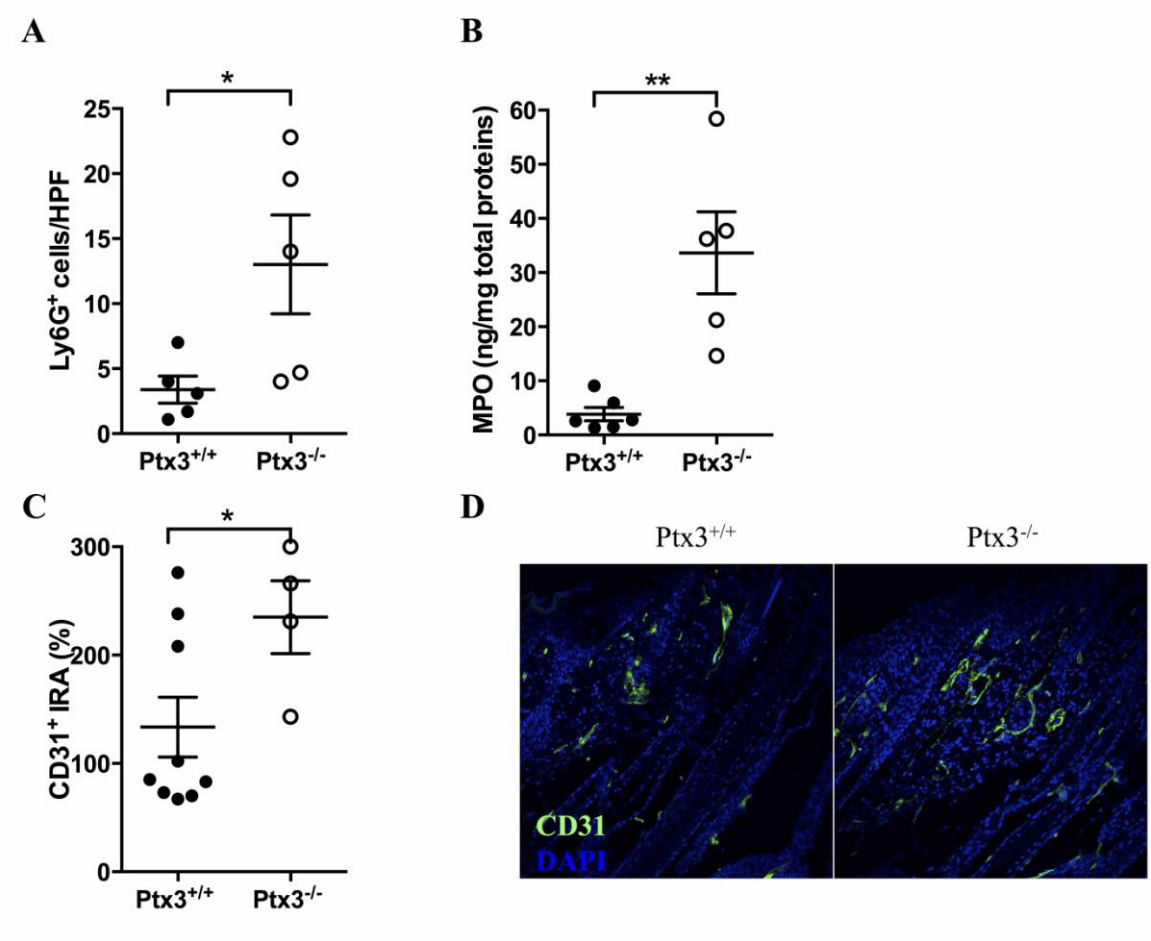

Fig. 24. PTX3-deficiency is associated with increased CRI in early carcinogenesis.

(A) Immunohistochemical analysis of neutrophil (Ly6G+) infiltration on PTX3-deficient and PTX3-competent skins. Mice are administered with DMBA/TPA for three weeks. Skin samples are collected 48 hours after the last TPA. Each dot represents the average of ten fields while the bar indicates the mean ± SEM. (B) MPO dosage by ELISA of skin homogenates from Ptx3$^{-/-}$ and Ptx3$^{+/+}$ mice (mean ± SEM). (C,D) Analysis of angiogenesis (CD31^{+} immunoreactive area, IRA) in cutaneous early lesions by immunofluorescence. Each dot represents the average of ten fields while the bar indicates the mean ± SEM . *: $p<0.05$, **: $p<0.01$, unpaired Student's t test.

Moreover, the concentration of pro-inflammatory cytokines IL-1α, IL-23 and the neutrophil attractant CXCL2 chemokine were significantly higher in Ptx3$^{-/-}$ than in Ptx3$^{+/+}$ skin homogenates (Fig. 25). Therefore, these data show that the lack of PTX3 is associated with increased neutrophil infiltration, MPO content as well as angiogenesis in early carcinogenesis. Moreover, PTX3-deficiency also correlates with higher levels of pro-inflammatory mediators.

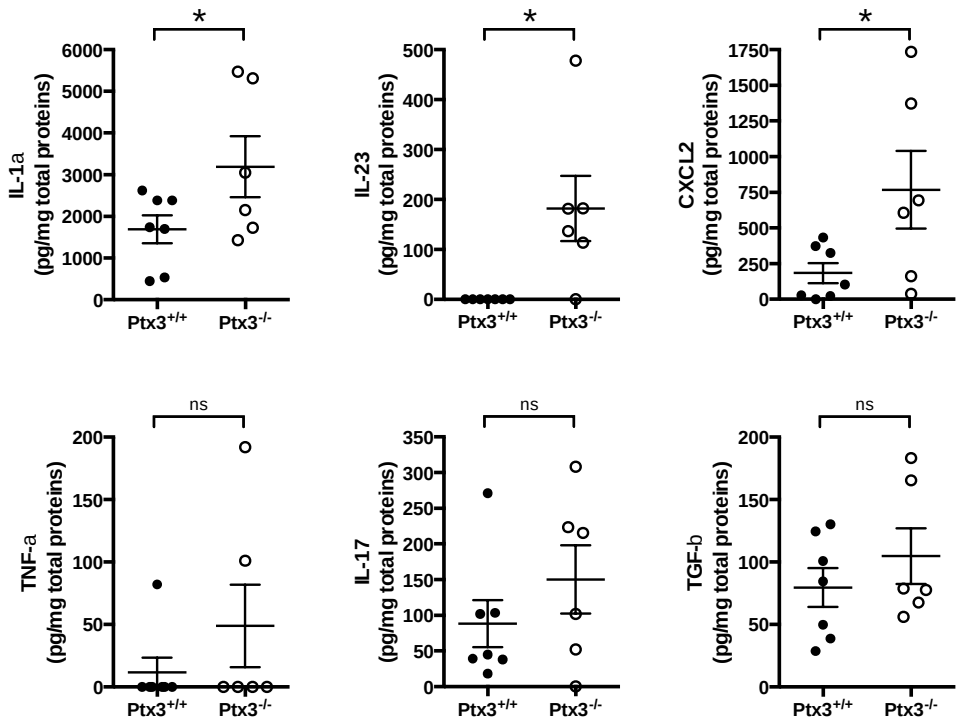

Fig. 25. PTX3-deficiency is associated with increased pro-inflammatory cytokines and chemokines in early carcinogenesis.
Analysis by ELISA of murine IL-1α, IL-23, TNF-α, IL-17, TGF-β and CXCL2 in skin homogenates from PTX3-deficient and PTX3-competent mice. Dosage of CCL2, IL-6, IL-12 and IFN-γ displays undetectable levels (data not shown). Mice are treated with DMBA/TPA for three weeks. Skin samples are collected 48 hours after the last TPA. (mean ± SEM). *: $p<0.05$, unpaired Student's t test.

4.4 PTX3-deficiency is associated with higher complement activation in the acute phase of carcinogenesis

In addition to promoting complement activation during microbial infections, as described for *A. fumigatus* infection[145], in sterile inflammation, including postischemic acute kidney injury[156] or myocardial stroke[150], PTX3 acts as a negative regulator of inflammation favoring FH activity. In 3-MCA induced fibrosarcoma $Ptx3^{-/-}$ lesions displayed higher C3 deposition than $Ptx3^{+/+}$, likely through the missing interaction of PTX3 with FH. Thereby, we focused on complement activation in the murine model of DMBA/TPA-induced skin cancer. Since C3 represents the central element of the three complement pathways, we determined C3 deposition in both early and late carcinogenesis. Interestingly, as shown in Fig. 26A, PTX3-deficiency was associated

with increased cutaneous C3 levels after three weeks of DMBA/TPA treatments, compared to $Ptx3^{+/+}$ treated-skins (p=0.04). In contrast, confocal analysis of C3 did not show significant differences between $Ptx3^{+/+}$ and $Ptx3^{-/-}$ papillomas in late carcinogenesis (Fig. 26B).

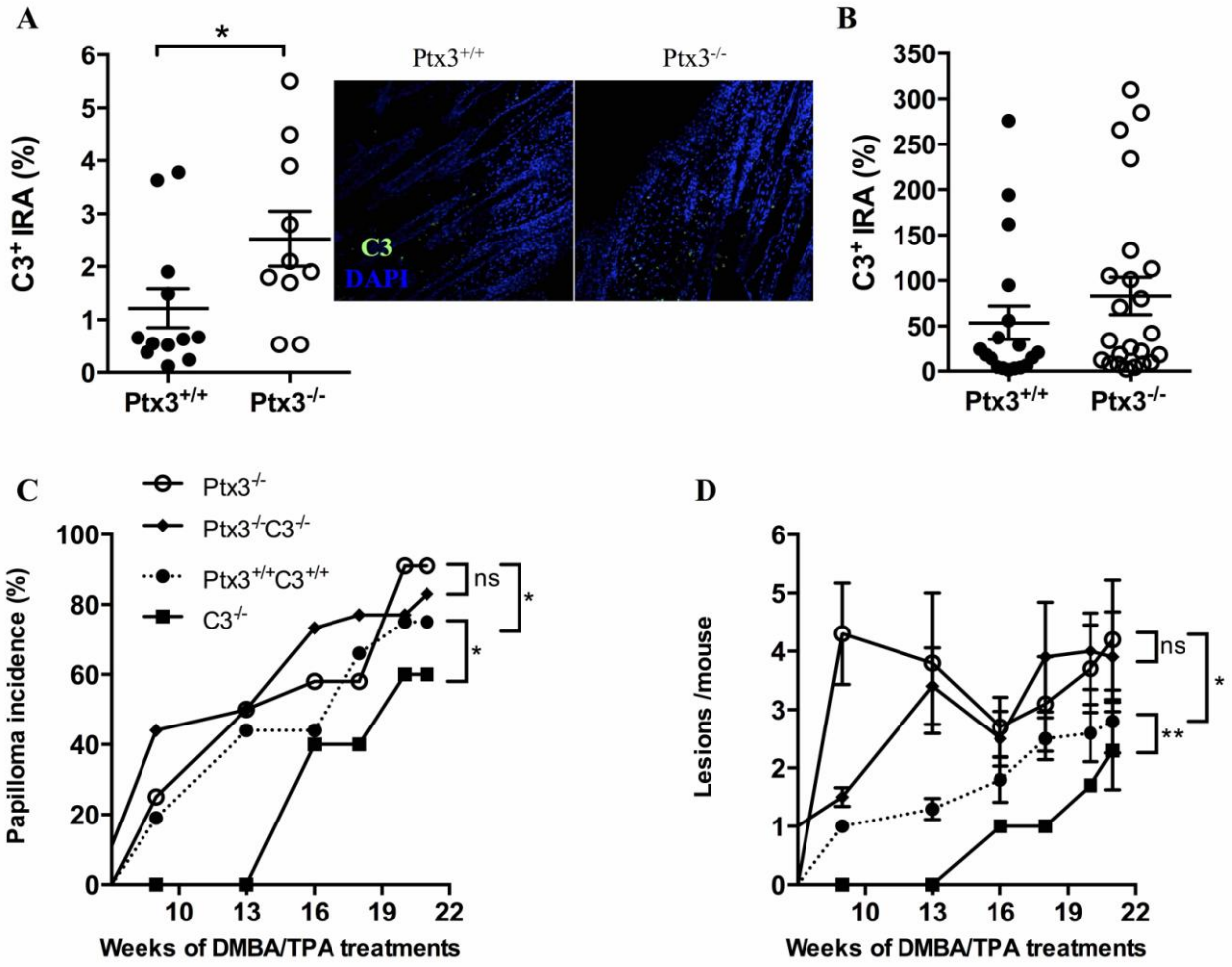

Fig. 26. PTX3-deficiency is associated with higher complement activation in early carcinogenesis. (A) Confocal microscopy of C3 deposition on PTX3-deficient and PTX3-competent skins. $Ptx3^{-/-}$ and $Ptx3^{+/+}$ mice are administered with DMBA/TPA for three weeks. Skin samples are removed 48 hours after the last TPA. (B) Confocal microscopy of C3 deposition on PTX3-deficient and PTX3-competent papillomas removed after 22 weeks of DMBA/TPA. Each dot represents a single field (mean ± SEM). *: $p<0.05$, unpaired Student's t test. (C, D) Incidence and mean number of papillomas, respectively, in C3 deficient and PTX3/C3-deficient mice. One experiment with 16 $C3^{+/+}$/$PTX3^{+/+}$, 11 $Ptx3^{-/-}$, 18 $C3^{-/-}$/$PTX3^{-/-}$ and 6 $C3^{-/-}$ mice of 6-10 wks. *: $p<0.05$, **: $p<0.01$, paired Student's test.

To assess the role of complement in the phenotype of PTX3-deficient mice, we evaluated the tumor susceptibility of C3 deficient and PTX3/C3- double deficient mice in DMBA/TPA-induced skin cancer.

As shown in Fig. 26C and D, C3-deficiency conferred protection in terms of both papilloma number and papilloma incidence compared to $Ptx3^{+/+}$ mice (p=0.0038; p=0.03). These data indicate the role of complement in tumor-promoting inflammation in both mesenchymal and epithelial tumor models. However, in this preliminary experiment, C3-deficiency did not reduce the tumor susceptibility of $Ptx3^{-/-}$ mice at the end of carcinogenesis (22 weeks). Then, these results suggest that the exacerbated inflammation of $Ptx3^{-/-}$ papillomas is partially dependent on the effect of complement.

4.5 PTX3-deficiency is associated with increased IgG1 levels in both early and late carcinogenesis

In a genetic model of murine squamous carcinogenesis in which the early region genes of human papillomavirus type 16 (HPV16) are under control of skin keratin 14, Coussens et al. have demonstrated the protumorigenic role of immunoglobulins (Igs) in CRI through FcγR-dependent mast cell and neutrophil recruitment[112]. PTX3 acts as a functional ancestor of antibodies and plays opsonic activity via FcγR, possibly competing with Igs[133].

Thereby, we investigated IgG deposition during DMBA/TPA-induced carcinogenesis. Interestingly, confocal microscopy showed higher IgG1 levels in PTX3-deficient skins obtained after three weeks of DMBA/TPA treatments, compared to PTX3-competent skins (Fig. 27A). Increased IgG1 deposition was also observed in $Ptx3^{-/-}$ papillomas taken during late carcinogenesis (30 weeks of DMBA/TPA treatments) compared $Ptx3^{+/+}$ papillomas (Fig. 27B). Further studies are needed to understand whether the increase of antibodies is responsible for higher inflammation of $Ptx3^{-/-}$ mice in skin carcinogenesis and whether FcγRs are involved in increased inflammation associated with carcinogenesis in PTX3-deficient mice.

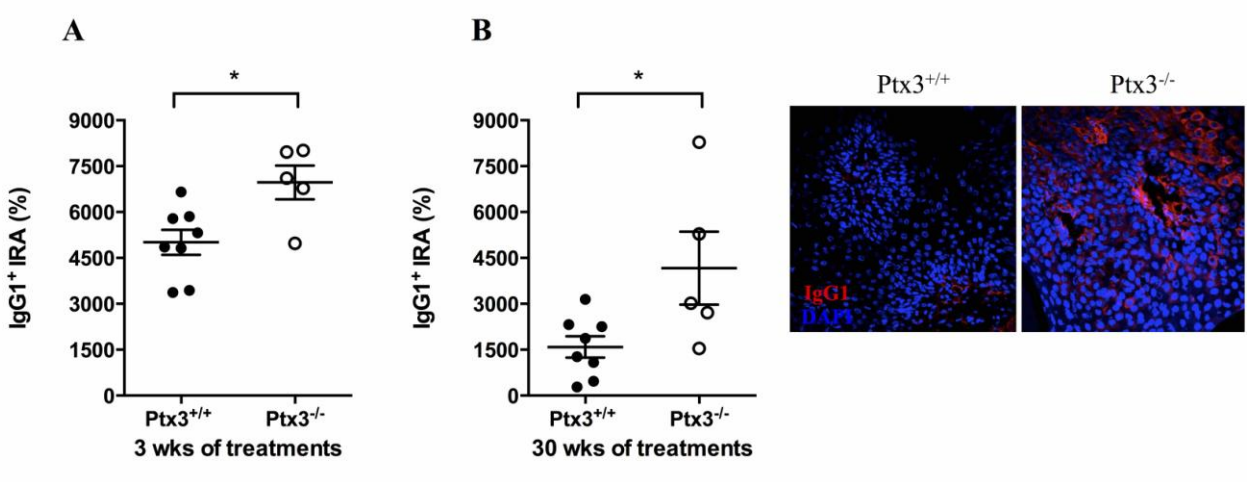

Fig. 27. PTX3-deficiency is associated with increased IgG1 levels in both early and late carcinogenesis. Analysis by confocal microscopy of IgG1 levels in both short-term treated skins and papillomas, respectively, from PTX3-deficient and competent mice (each dot represents the average of ten fields and the bar indicates the mean ± SEM) *: $p<0.05$, unpaired Student's t test.

4.6 PTX3-deficiency contributes to increased neutrophil infiltration in late carcinogenesis

Several data from the literature indicate the protumorigenic role of neutrophils in DMBA/TPA-induced skin cancer[56]. Immunohistochemical analysis displayed increased neutrophil infiltration ($Ly6G^+$ cells) of $Ptx3^{-/-}$ papillomas, collected 48 hours after 16 weeks of DMBA/TPA, compared to $Ptx3^{+/+}$ papillomas (Fig. 28A), similarly to what observed after short-term TPA treatments.

As shown in Fig. 28B and C, macrophage ($F4/80^+$) and T-lymphocyte ($CD3^+$) infiltration did not significantly differ between $Ptx3^{-/-}$ and $Ptx3^{+/+}$ papillomas, although the number of samples should be increased.

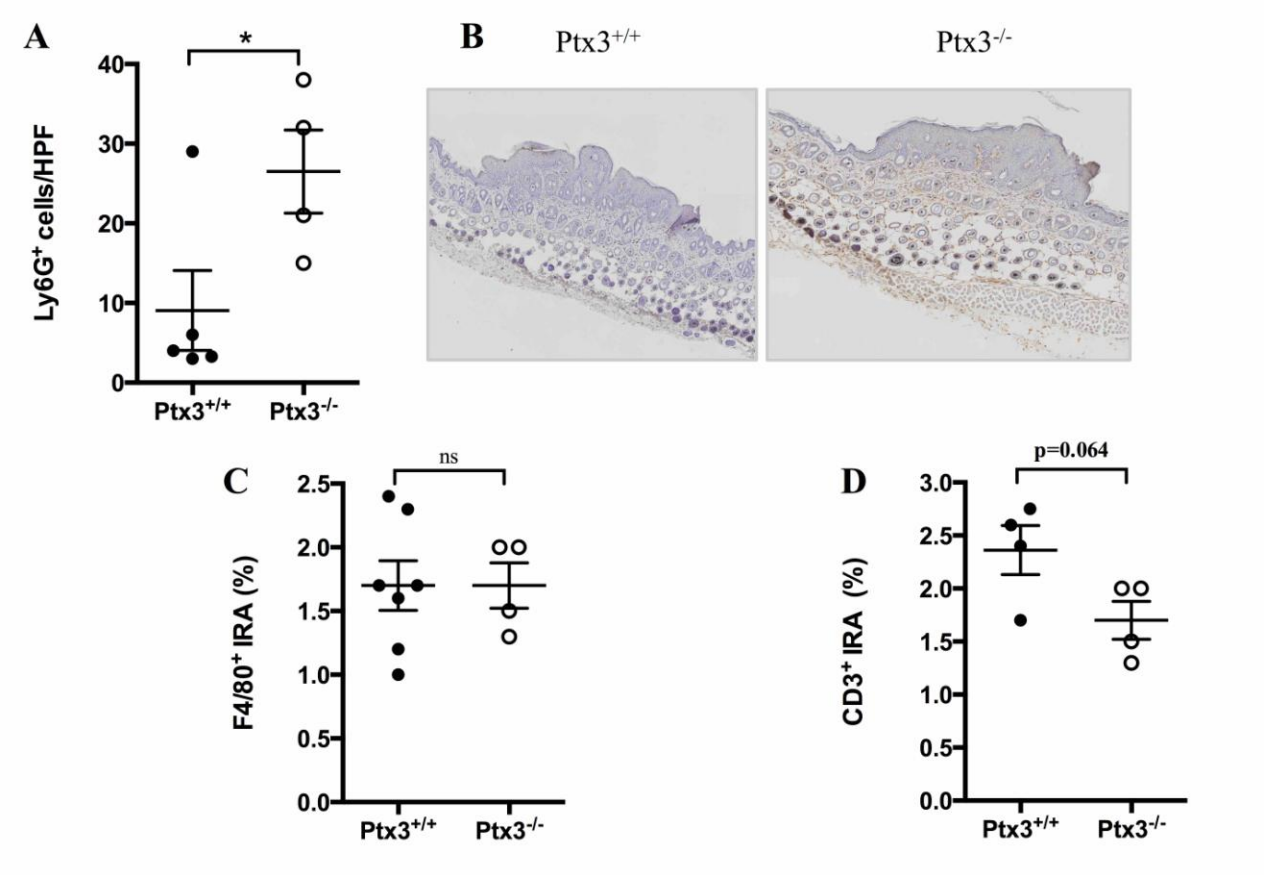

Fig. 28. PTX3-deficiency is associated with higher CRI in late carcinogenesis. (A-B) Immunohistochemical analysis of neutrophil (Ly6G) in PTX3-deficient and PTX3-competent papillomas. (C-D) Immunohistochemical analysis of macrophage (F4/80) and T-cell (CD3) infiltration. Ptx3-deficient and Ptx3-competent mice are administered with DMBA/TPA for 16 weeks. Skin samples are collected 48 hours after the last TPA (each dot represents the average of ten fields, while the bar shows mean ± SEM). *: $p<0.05$, unpaired Student's t test.

Furthermore, FACS analysis of circulating leukocytes from PTX3-deficient and competent mice treated with DMBA/TPA for 22 weeks displayed high level of neutrophils associated with PTX3-deficiency (Fig. 29). In addition, $Ptx3^{-/-}$ mice exhibited increased circulating levels of $CD4^+$ T cell compared to $Ptx3^{+/+}$ mice. The capacity of $CD4^+$ T-cells to favor the development of tumors is not without precedent. Yusuf et al. have demonstrated that $CD4^{-/-}$ mice developed less papillomas than immunocompetent mice in DMBA cutaneous carcinogenesis[26]. However, the higher number of circulating $CD4^+$ T cells associated with PTX3-deficiency is unexpected and not explained by knowledge of PTX3 function. The percentage of monocytes ($CD11b^+CD115^+$ cells), DCs ($CD11c^+$cells), NK cells ($NK1.1^+$ cells), CTL ($CD8^+$ cells) and B-cells ($CD19^+$ cells) was comparable in the two groups (Fig. 29).

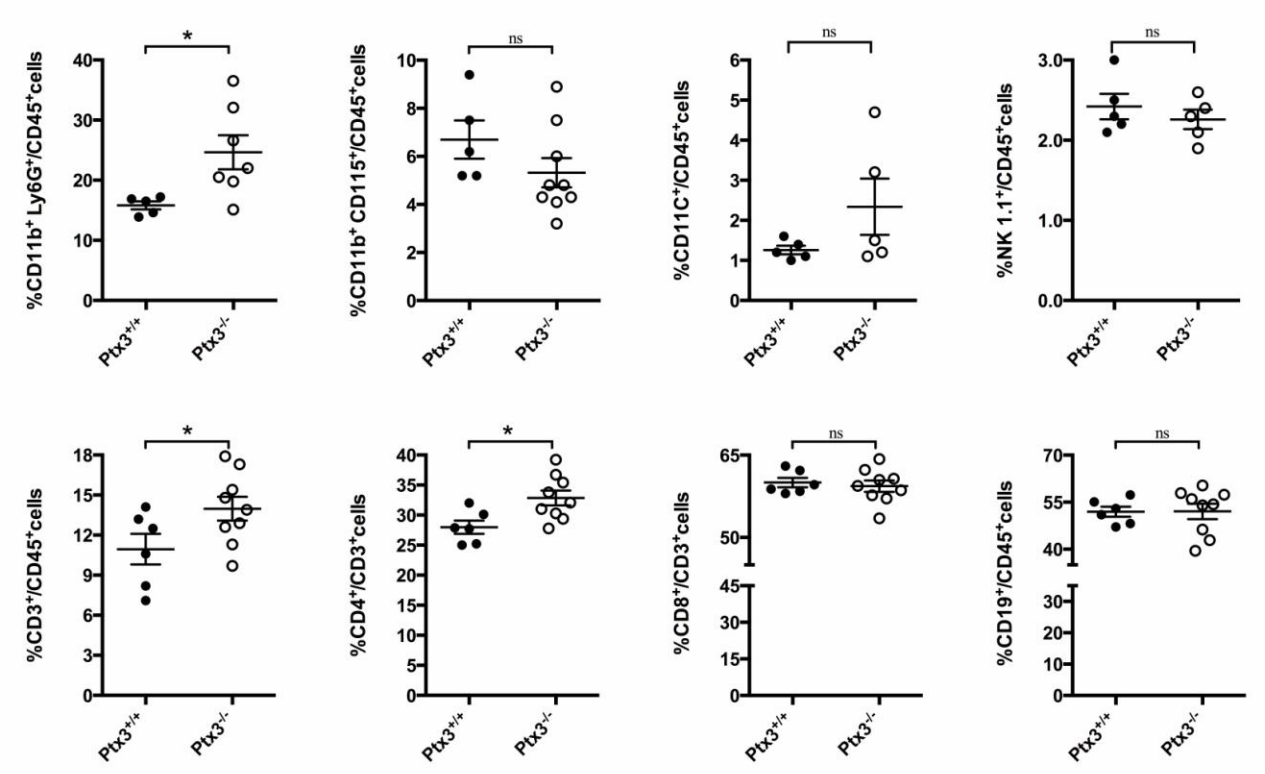

Fig. 29. FACS analysis of circulating leukocytes from PTX3-deficient and competent mice in late carcinogenesis. FACS analysis of circulating leukocytes from PTX3-deficient and competent mice treated with DMBA/TPA for 22 weeks. Blood samples are collected 48 hours after the last TPA (mean ± SEM). *: $p<0.05$, unpaired Student's t test.

4.7 The protective role of PTX3 through the modulation of P-selectin-dependent neutrophil recruitment

Since in DMBA/TPA chemically induced cancer the higher complement activation associated with PTX3-deficiency is not totally responsible for increased susceptibility to SCC, we have investigated a further mechanism used by PTX3 to modulate inflammation. PTX3 has been shown to regulate P-selectin-dependent neutrophil recruitment[151]. We thus investigated the tumor susceptibility of P-selectin$^{-/-}$ and PTX3/P-selectin double deficient mice in DMBA/TPA-induced skin carcinogenesis. As shown in Fig. 30 A-B, P-selectin$^{-/-}$ mice were strongly protected in terms of number and incidence of papillomas compared to immunocompetent mice after 22 weeks of DMBA/TPA treatments ($p<0.0001$; $p<0.0011$ respectively). Interestingly, P-selectin-deficiency

reverted the phenotype of Ptx3$^{-/-}$ mice to the level of P-selectin$^{-/-}$ mice in terms of both number and incidence of papillomas.

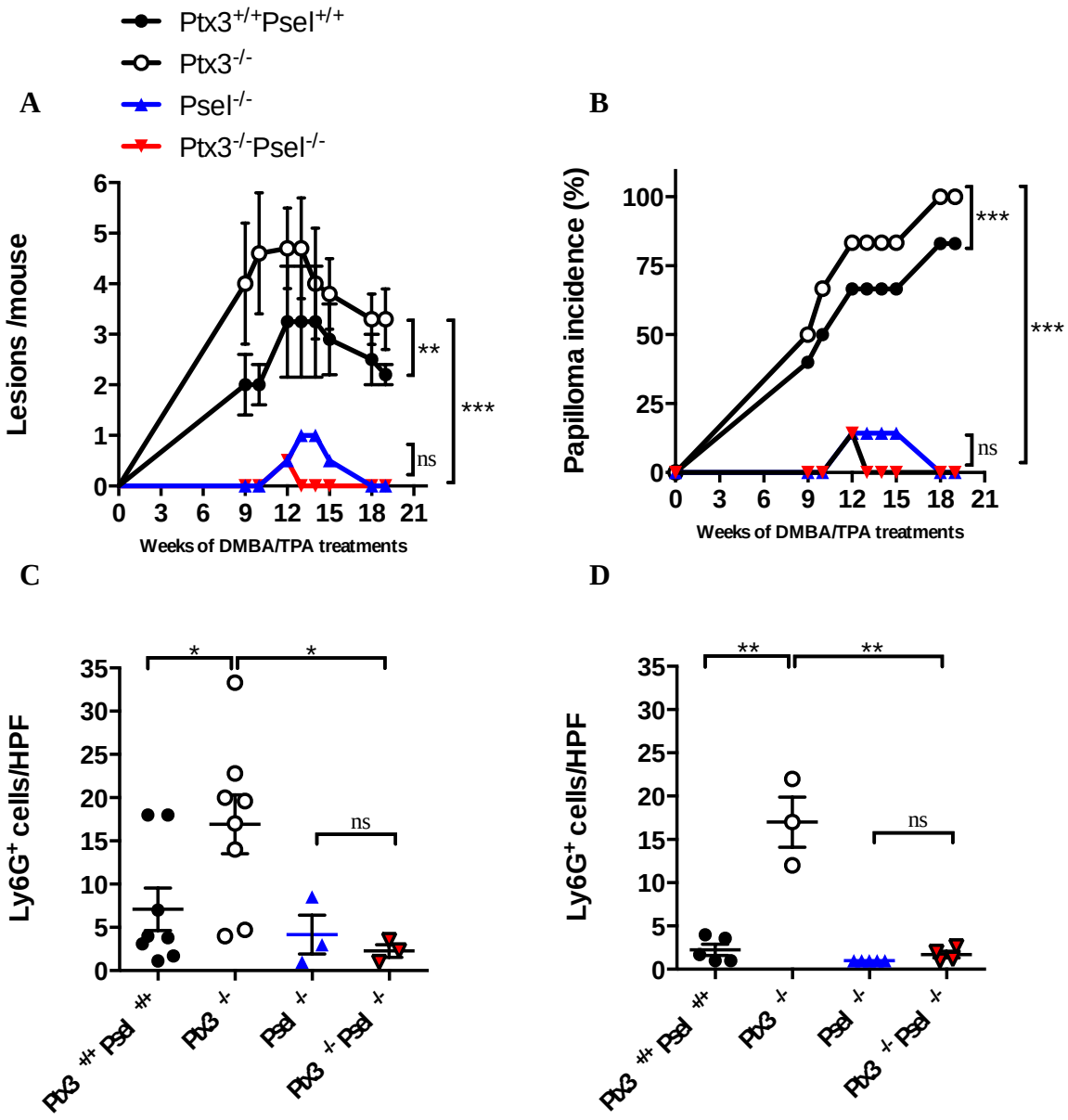

Fig. 30. P-selectin-deficiency reverts the tumor susceptibility of Ptx3$^{-/-}$ mice. Results are shown as (A) number of papillomas per mouse and (B) percentage of papilloma incidence in each group (mean ± SEM). Groups of female Ptx3$^{+/+}$/Pselectin$^{+/+}$, Ptx3$^{-/-}$, P-selectin$^{-/-}$ and Ptx3$^{-/-}$P-selectin$^{-/-}$ mice are treated with 25 µg DMBA, and 1 week later, thrice weekly with 4 µg TPA for 22 weeks. One experiment with 6 mice/group is shown. **: $p<0.01$, ***: $p<0.001$, paired Student's t test. (C) Immunohistochemical analysis of neutrophil (Ly6G+) infiltration in the skin. Groups of female Ptx3$^{+/+}$/Pselectin$^{+/+}$, Ptx3$^{-/-}$, P-selectin$^{-/-}$ and PTX3/P-selectin-double deficient mice are administered with DMBA/TPA for three weeks. Skin samples are collected 48 hours after the last TPA. (D) Immunohistochemical analysis of neutrophil (Ly6G+) infiltration in papilloma recovered after 22 weeks of DMBA/TPA. Each dot represents the average of ten fields. *: $p<0.05$, **: $p<0.01$, unpaired Student's t test.

Moreover, immunohistochemical analysis of both short-term DMBA/TPA treated skins and papilloma, respectively, demonstrated a marked reduction of neutrophils (Ly6G+) associated to P-selectin$^{-/-}$ and Ptx3$^{-/-}$/P-selectin$^{-/-}$ compared to Ptx3$^{-/-}$ treated-skins (Fig. 30C-D). As shown in Fig. 31, FACS analysis of blood cells after 22 weeks of DMBA/TPA indicated comparable percentage of leukocytes between P-selectin-deficient and Ptx3/P-selectin double deficient mice. P-selectin-deficiency was associated with a circulating neutrophil accumulation possibly related to the inability of these cells to reach the tissues. In addition, low levels of circulating CD4^{+} T cells and CD19^{+} B cells were observed in P-selectin$^{-/-}$ mice.

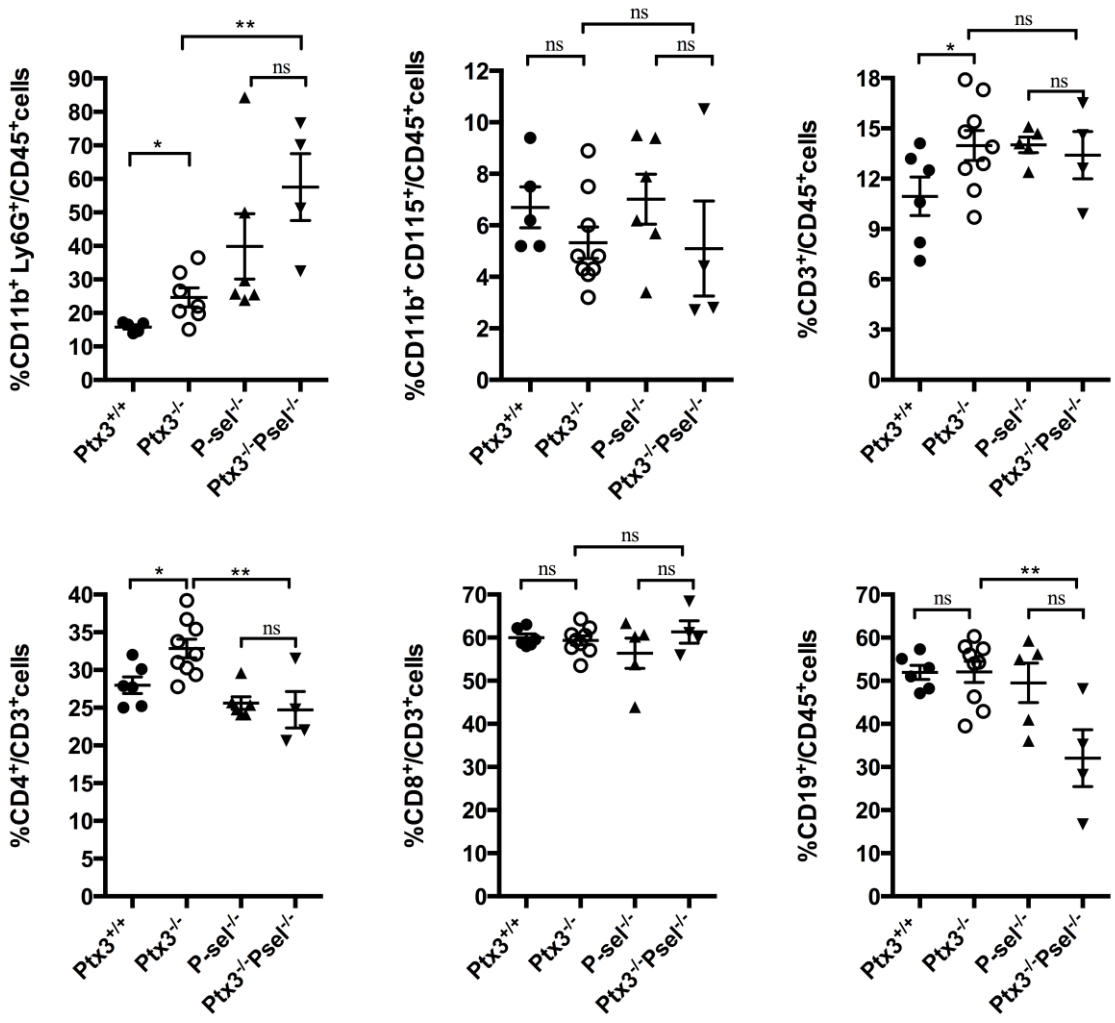

Fig. 31. FACS analysis of circulating leukocytes from P-selectin deficient and PTX3/P-selectin double deficient mice in late carcinogenesis. FACS analysis of total leukocytes. Ptx3-, P-selectin- and PTX3/P-selectin-deficient and immunocompetent mice are treated with DMBA/TPA for 22 weeks. Blood samples are collected 48 hours after the last TPA (mean ± SEM). *: $p<0.05$, unpaired Student's t test.

Although P-selectin-deficiency did not affect the tumor susceptibility of $Ptx3^{-/-}$ mice in 3-MCA-carcinogenesis, our results suggest the tumor-promoting role of P-selectin in DMBA/TPA-induced skin carcinogenesis possibly favoring an exacerbated neutrophil recruitment. Then, PTX3 plays a protective role in skin carcinogenesis as results of modulation of P-selectin-dependent neutrophil recruitment.

4.8 The in vivo neutrophil depletion reverts the tumor susceptibility of PTX3-deficient mice

To assess the role of neutrophils in increased carcinogenesis to DMBA/TPA-induced skin carcinogenesis exhibited by $Ptx3^{-/-}$ mice, we treated both PTX3-competent and deficient mice with an anti-Ly6G antibody throughout the DMBA/TPA experiment.

As shown in Fig. 32A, neutrophil depletion did not affect the papilloma multiplicity of immunocompetent mice, whereas it decreased the susceptibility of $Ptx3^{-/-}$ mice, reverting the phenotype. FACS analysis of blood cells, performed at the end of carcinogenesis, confirmed neutrophil depletion and did not show differences of circulating monocytes among groups (Fig. 32B-C).

Data obtained by P-selectin-deficient mice and Ly6G depletion, both dampening neutrophil infiltration, suggest the relevance of these cells in the exacerbated inflammation shown in $Ptx3^{-/-}$ papillomas.

Finally, we also used a genetic approach to address the relevance of neutrophils in this model. We treated both GCSFR-competent and deficient mice with DMBA/TPA for 22 weeks. CSF3R is the transmembrane receptor, for colony stimulating factor 3 (G-CSF), a cytokine that controls the production, differentiation, and function of granulocytes[168]. As shown in Fig. 32D-E, $Csfgr^{-/-}$ mice were protected in terms of both papilloma number and incidence ($p<0.01$; $p<0.003$, respectively).

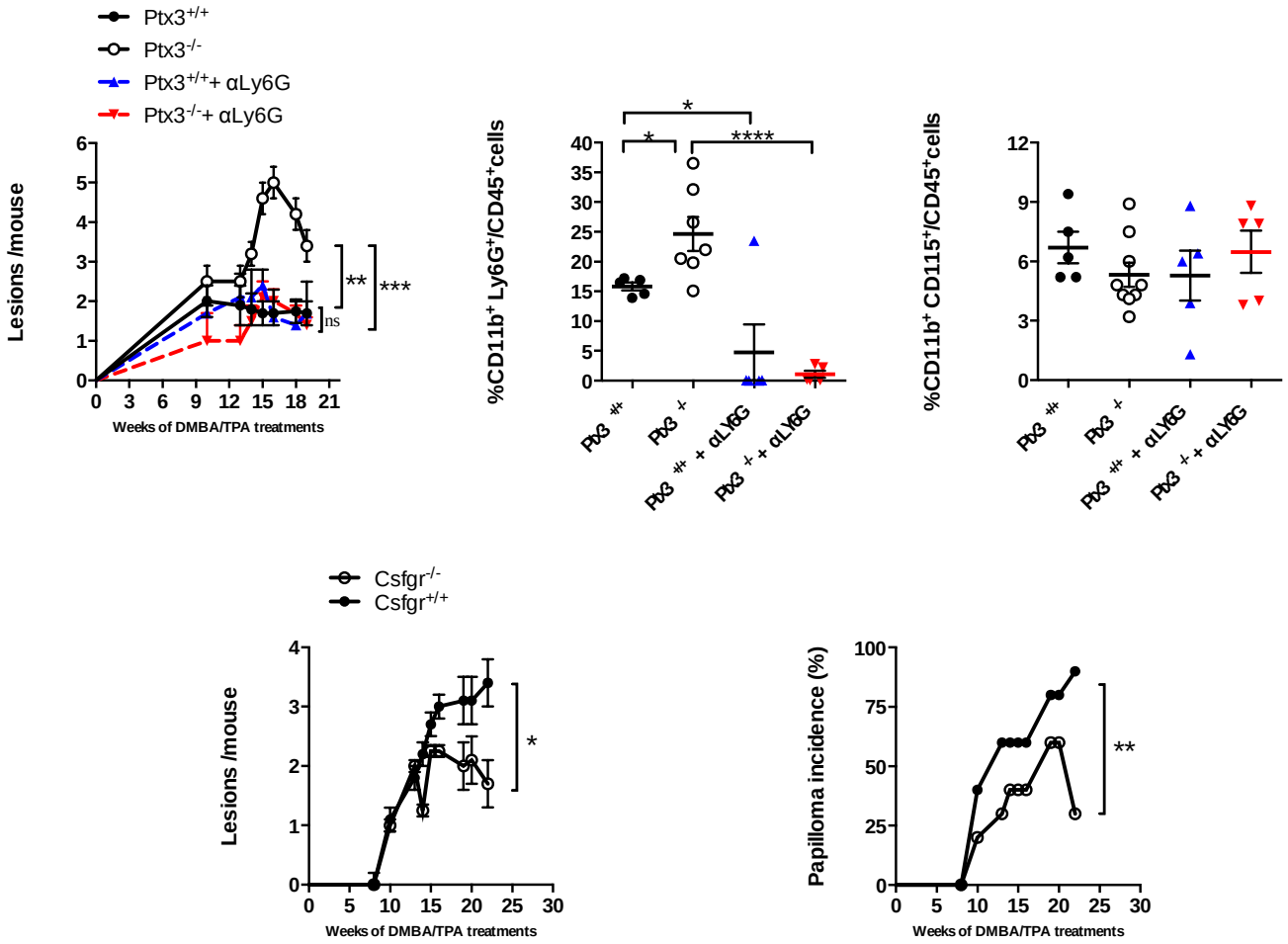

Fig. 32. Role of neutrophils in skin carcinogenesis. (A) Number of DMBA/TPA-induced papillomas in Ptx3$^{-/-}$ and Ptx3$^{+/+}$ mice treated with anti-Ly6G, or isotype mAb, twice weekly for 22 weeks throughout the DMBA/TPA experiment (results shown as mean ± SEM). One experiment with 6 mice/group. **: p<0.01, ***: p<0.001, paired Student's t test. (B, C) FACS analysis of circulating neutrophils (Ly6G^{+}) and monocytes (CD11b^{+}CD115^{+}) at 22 weeks of DMBA/TPA (mean ± SEM). *: p<0.05, ****: p<0.001, unpaired Student's t test. (D, E) Percentage of papilloma number and incidence in each group (mean ± SEM). Groups of 10 female Csfgr$^{-/-}$ and Csfgr$^{+/+}$ mice are treated with 25 µg DMBA, and 1 week later, three times weekly with 4 µg TPA for 22 weeks. **: p<0.01, *: p<0.05, paired Student's t test.

FACS analysis of circulating leukocytes from CSFGR-deficient and competent mice treated with DMBA/TPA for 22 weeks displayed a severe neutropenia associated with CSFGR-deficiency (Fig. 33). In addition, Csfgr$^{-/-}$ mice exhibited high circulating levels of CD8^{+} T cells, which were known to play a protective role in skin tumor development[26].

All together these approaches demonstrate the pro-tumorigenic role of neutrophils in chemically induced SCC.

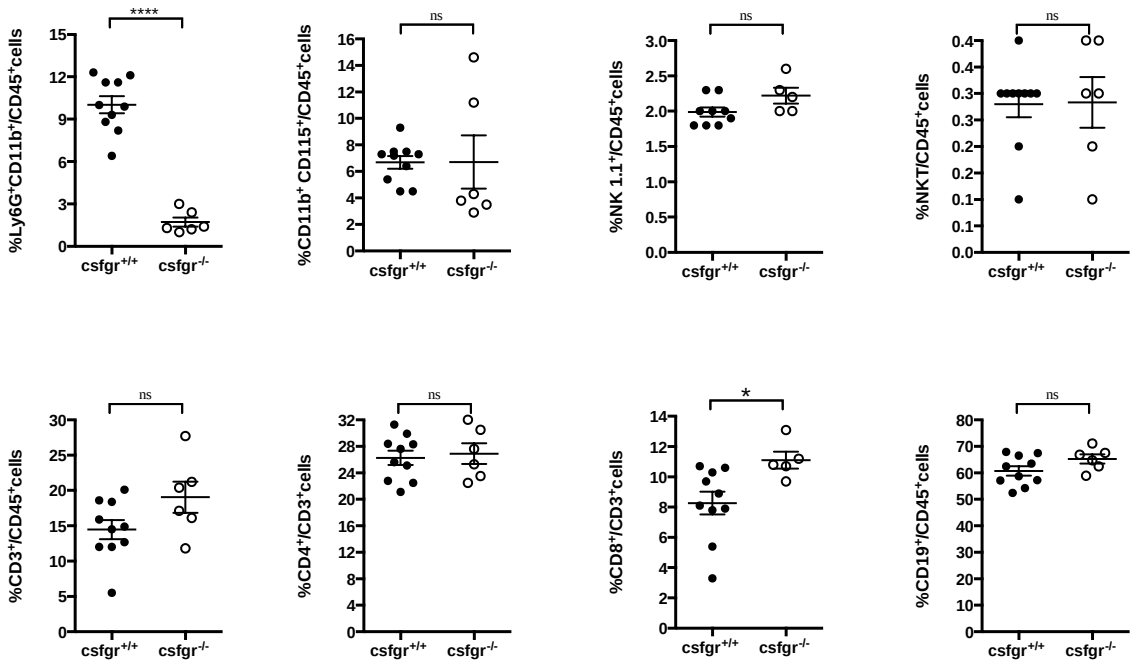

Fig. 33. FACS analysis of circulating leukocytes from CSFGR deficient and competent mice in late carcinogenesis. FACS analysis of total leukocytes. CSFGR-deficient and immunocompetent mice are treated with DMBA/TPA for 22 weeks. Blood samples are collected 48 hours after the last TPA (mean ± SEM). *: $p<0.05$, ****: $p<0.001$, unpaired Student's t test.

5 Materials and methods

5.1 Animals. Wild-type C57BL/6J male mice were obtained from Charles River Laboratories, Calco, Italy. PTX3-deficient mice were generated by Garlanda et al.[130]. P-Selectin$^{-/-}$, C3$^{-/-}$ and Gcsfr$^{-/-}$ mice were purchased from The Jackson Labs, Bar Harbor ME, USA. PTX3/C3 and PTX3/P-selectin double deficient mice were generated by crossing PTX3- and C3- or P-selectin deficient mice. All mice were used on a C57BL/6J genetic background. Mice were housed in the SPF animal facility of Humanitas Clinical and Research Center in individually ventilated cages. Procedures involving animals handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). The study was approved by Italian Ministry of Health (approval n. 71/2012-B, issued on the 09/03/2012). All efforts were made to minimize the number of animals used and their suffering.

5.2 Carcinogenesis model. Skin carcinogenesis was performed as reported (Schioppa et al., 2011) in a two-step DMBA/TPA model. Initiation was accomplished by pipetting 25μg of DMBA (Sigma-Aldrich) in acetone onto shaved back skin of 6-7-weeks old female mice. The target age for beginning DMBA/TPA skin carcinogenesis were 6-7 weeks of age because most of mice are in the resting phase of hair cycle. Promotion, begun 1 week after the initiation, was performed three times a week with 4μg of TPA (Sigma-Aldrich) from week 1 to week 30. During the experiment, mice were monitored weekly for papilloma/carcinoma development. Only palpable tumors larger than 1 mm or present for at least a week were counted. Papilloma started to develop from the eighth or ninth week. Papilloma converted to SCC after at least 20

weeks of promotion[117]. Four experiments with 10 female $Ptx3^{-/-}$ and $Ptx3^{+/+}$ mice and one experiment with 6-18 female P-$selectin^{-/-}$, $C3^{-/-}$, $Cfs3r^{-/-}$, $PTX3^{-/-}/C3^{-/-}$ and $Ptx3^{-/-}$/P-$selectin^{-/-}$ mice were performed.

5.3 Blocking of Ly6G *in vivo*. 6 $Ptx3^{+/+}$ and 6 $Ptx3^{-/-}$ mice were treated one day before DMBA treatment with 200µg of specific Ab (control rat IgG2A, Clone 2A3; or rat anti-Ly6G, Clone 1A8) and than every three days with 100µg for the entire duration of the experiment (22 weeks). To evaluate neutrophil/Ly6G+ cell depletion, 200µl blood was collected by retro-orbital sinus of anesthetized mice into EDTA-containing tubes at the second week and the end of DMBA/TPA treatment. Red blood cells were lysed in 0.8% NH4Cl, 0.1 mM EDTA for 20 minutes on ice. Then, lysed blood samples were washed twice in $PBS^{-/-}$ 1X and centrifuge at 2500 rpm for 20 min at 4°C. Plasma was removed and the pellets were stained in the dark for 30 min at 4° C with fluorescent Ab against CD45 (CD45-BV605; clone 30-F11), CD11b (CD11b-BV421; clone M1-70), Ly6G (Ly6G PE-CF-594, clone 1A8) and CD115 (CD115-APC, clone AFS98) from BD Bioscience, eBioscience or BioLegend in 50µl FACS buffer (2% FBS, 2mM EDTA, 0,05% NaN_3 in $PBS^{-/-}$ 1X). Cells were washed in FACS buffer and fixed with 300µl formalin (2%) in $PBS^{-/-}$ 1X. Cells were analysed by cytometry on LSR Fortessa (BD Bioscience).

5.4 ELISA. 5 untreated or vehicle (acetone) skins and cutaneous samples treated 1 or 5 times with DMBA/TPA, respectively, were recovered 12 hours after the last TPA from $Ptx3^{+/+}$ mice. Moreover, $Ptx3^{+/+}$ papillomas were removed 24 hours after 16 weeks of DMBA/TPA. Skin samples or papillomas were homogenized in 1ml PBS with Ca^{2+}/Mg^{2+} ($PBS^{+/+}$) containing protease inhibitors (Complete®-EDTA-free; Roche Diagnostics) and PMSF (1mM). Tissue

homogenates were centrifuged at 14000 rpm for 30 min at 4 °C. Then, supernatants were quantified by Coomassie plus protein assay reagent (Thermo Scientific) and stored at -20 °C for PTX3 analysis by ELISA (R&D DuoSet ELISA Development System) according to manufacturer's instructions. Skin samples treated for 3 weeks with DMBA/TPA and recovered 24 hours after the last TPA, from 7 $Ptx3^{+/+}$ and 6 $Ptx3^{-/-}$ mice, were homogenized, and quantified, as described above, for cytokine analysis. Murine IL-1α, IL-23, TNF-α, IL-17, TGF-β, CXCL2, CCL2, IL-6, IL-12, IFN-γ and MPO were measured in tissue homogenates by ELISA (R&D DuoSet ELISA Development System) according to manufacturer's instructions.

5.5 FACS analysis. Blood was collected by retro-orbital sinus of 6 anesthetized experimental groups (6 $Ptx3^{+/+}$/P-$selectin^{+/+}$, 9 $Ptx3^{-/-}$, 6 P-selectin-/-, 6 $Ptx3^{-/-}$/P-$selectin^{-/-}$ mice, 10 $Gcsfr^{+/+}$ and 6 $Gcsfr^{-/-}$), after 22 weeks of DMBA/TPA treatments, into EDTA-containing tubes. Red blood cells were lysed in 0.8% NH4Cl, 0.1 mM EDTA for 20 minutes on ice. Then, lysed blood samples were washed twice in $PBS^{-/-}$ 1X and centrifuge at 2500 rpm for 20 min at 4°C. Plasma was removed and the composition of leukocytes in the blood was determined by flow cytometry using the combination of following antibodies: CD45-BV605 (clone 30-F11); CD3e-FITC (clone 145-2C11); CD8-PE (clone 53-6.7); CD4-Alexa700 (clone RM4-5); CD19-Pacific Blue (clone 1D3); Ly6G PE-CF-594 (clone 1A8); CD11b-BV421 (clone M1-70); CD115-APC (clone AFS98); CD11c-Alexa700 (clone N418); T cells/Not CD4 Not CD8 NK1.1-APC (clone PK136) from BD Bioscience, eBioscience or BioLegend. NKT cells were characterized as $CD3^{+}CD4^{-}$ $CD8^{-}$ T cells. Cells were analysed on LSR Fortessa (BD Bioscience).

5.6 Haematoxylin-Eosin (H&E) At least 10 samples from $Ptx3^{+/+}$ and $Ptx3^{-/-}$ OCT-embedded mouse papillomas recovered after 16 or 30 weeks of DMBA/TPA treatments were analysed.

Skin tissues were fixed in 4% PFA, washed twice in PBS, dehydrated in 20, 30 and 40% sucrose solutions gradient, mounted in OCT embedding compound and stored at -80°C. Eight-µm consecutive sections were cut from the middle of the tissue and used for histological examination in each mouse. Sections for histological analysis were fixed in 4% formalin and stained with haematoxylin (2-3 min) and eosin (10 dives). Then, sections were dehydrated in increasing concentrations of alcohols (70%-90%-100%-100%), clarified in xylene and analysed by a pathologist.

5.7 Immunohistochemistry. Skin samples treated one time with DMBA/TPA were recovered 12 hours after the last TPA from $Ptx3^{+/+}$ mice. $Ptx3^{+/+}$ papillomas were removed 24 hours after 16 weeks of DMBA/TPA. $Ptx3^{-/-}$ tissues were used as negative control. Then, treated-skins or papillomas were fixed in 4% PFA, washed twice in PBS, dehydrated in 20, 30 and 40% sucrose solutions gradient, mounted in OCT embedding compound and stored at -80°C. Eight-µm consecutive sections were cut from the middle of the tissue and used for PTX3 immunohistochemistry. Endogenous peroxidase was blocked for 5 min with a Peroxidized 1 (Biocare Medical) ready to use. Unspecific sites were blocked with Rodent Block M (Biocare Medical) 30 min and tissues were incubated for two hours with affinity-purified Ig against PTX3 (rabbit polyclonal IgG) in PBS supplemented with BSA (2%) and NP40 (0.02%). Envision^{+} System HRP Labelled Polymer anti Rabbit (Dako) was used as secondary antibody for 30 minutes. After washing, slides were developed with DAB (3,3'-diaminobenzidine) (Biocare Medical) and counterstained with Hematoxilyn. Tissues were dehydrated with ethanol, mounted with Eukitt and analysed with an Olympus BX61 virtual slide scanning system.

Skin samples collected 24 hours after 3 weeks of DMBA/TPA and papillomas obtained 24 hours after 16 weeks of DMBA/TPA, from $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice, were processed, as described

above, and stained for one hour with high affinity-purified Ig against F4/80 (rat-anti mouse; clone FA-11; Hycult® Biotech), Ly6G (rat-anti mouse; clone 1A8; BD Bioscience), CD3 (rat-anti human/mouse; clone CD3-12; AbD Serotec). Rat on Mouse HRP-Polymer kit (Biocare Medical) was used as secondary antibody in PBS supplemented with BSA (2%) and NP40 (0.02%). After washing, slides were developed with DAB (3,3'-diaminobenzidine) (Biocare Medical) and counterstained with Hematoxilyn. Tissues were dehydrated with ethanol, mounted with Eukitt and analysed with an Olympus BX61 virtual slide scanning system.

Skin samples collected 48 hours after 3 weeks of DMBA/TPA and papillomas obtained 24 hours after 22 weeks of DMBA/TPA, from $Ptx3^{+/+}$P-selectin$^{+/+}$, P-selectin$^{-/-}$, $Ptx3^{-/-}$, $Ptx3^{-/-}$P-selectin$^{-/-}$ mice, were processed, as described above, and stained for one hour with high affinity-purified Ig against Ly6G (rat-anti mouse; clone 1A8; BD Bioscience). Rat on Mouse HRP-Polymer kit (Biocare Medical) was used as secondary antibody in PBS supplemented with BSA (2%) and NP40 (0.02%). After washing, slides were developed with DAB (3,3'-diaminobenzidine) (Biocare Medical) and counterstained with Hematoxilyn. Tissues were dehydrated with ethanol, mounted with Eukitt and analysed with an Olympus BX61 virtual slide scanning system.

5.8 Immunofluorescence. Skin tissues treated with a single application of DMBA/TPA and recovered after eight hours from $Ptx3^{+/+}$ mice were fixed in PFA (4%), washed twice in PBS, dehydrated in 20, 30 and 40% sucrose solution gradient, mounted in OCT embedding compound and stored at -80°C. Eight-µm consecutive sections were cut, mounted on Superfrost slides and fixed in 4% PFA for 15 min, rehydrated in PBS and blocked in 5% normal goat (Dako, Denmark) or donkey (Sigma- Aldrich) serum, 2% BSA, 0.1% Triton® X-100 (Sigma-Aldrich) in $PBS^{+/+}$ pH 7.4 for 1h at room temperature. Sections were incubated with the following primary antibodies for 2h at room temperature: affinity purified rabbit polyclonal IgG anti-PTX3 or goat

IgG anti-mouse PTX3 (R&D Systems); rat anti-mouse F4/80 (Clone FA-11; Hycult® Biotech) or rat anti-mouse Ly6G (Clone 1A8; BD Bioscience). $Ptx3^{+/+}$ and $Ptx3^{-/-}$ papillomas obtained after 16 and 30 weeks of DMBA/TPA administration were processed, as described above, and stained with the following primary antibodies for 2h at room temperature: affinity purified goat polyclonal IgG anti-mouse PTX3 (R&D Systems) and rabbit polyclonal anti-mouse LYVE-1 (Abcam); FITC-conjugated goat F(AB')2 fragment to mouse Complement C3 (Cappel, MP biomedical); goat polyclonal anti-mouse IgG1 (Southern Biotech); rabbit anti-mouse Keratin 1 (AF109; Covance) and rabbit anti-mouse Keratin 14 (AF64; Covance), respectively. Skin samples taken after three weeks of treatments, were processed, as discussed, and stained with goat polyclonal anti-mouse IgG1 (Southern Biotech) or FITC-conjugated goat F(AB')2 fragment to mouse Complement C3 (Cappel, MP biomedical). After washing, sections were incubated for 1h with the following species-specific cross-adsorbed detection antibodies: Alexa® Fluor 488-conjugated donkey or goat anti-rabbit; Alexa® Fluor 647- conjugated donkey anti-goat by Invitrogen- Molecular Probes and Alexa® Fluor 594-conjugated donkey or goat anti-rat by Invitrogen-Molecular Probes. For DNA detection, DAPI (300nM; Invitrogen-Molecular Probes) was used. After each step, sections were washed with $PBS^{+/+}$ pH 7.4 containing 0.01% (v/v) Tween® 20. Sections were mounted with the antifade medium FluorPreserve Reagent (Calbiochem) and analysed with an Olympus Fluoview FV1000 laser scanning confocal microscope with 10X (N. A. 0.4). Negative controls were obtained by omission of the primary antibody or using $Ptx3^{-/-}$, $C3^{-/-}$ tissues.

5.9 Statistical analysis. All values were expressed as mean ± SEM. Unpaired Student's t test, paired Student's t test, chi-square test were used as specified. $p \leq 0.05$ was considered significant. Statistics were calculated with GraphPad Prism version 5, GraphPad Software.

Discussion

The general aim of this research was to question the role of the humoral arm of innate immunity in tumor-promoting inflammation using the long pentraxin PTX3 as a paradigm[128]. Previous observations by our group demonstrated increased susceptibility to 3-MCA-induced mesenchymal cancer in PTX3-deficient mice. This phenotype was associated with increased macrophage infiltration, cytokine production, angiogenesis, and genetic instability as revealed by an increased frequency of *Trp53* mutations, oxidative DNA damage and expression of DDR markers in $Ptx3^{-/-}$ mice. Several lines of evidence indicate that PTX3-deficiency triggers an exacerbated Complement activation, because of defective recruitment of the negative regulator Factor H, with higher production of C5a, CCL2 and increased number of tumor-promoting macrophages with an M2-like phenotype in 3-MCA-induced fibrosarcoma.

In selected human tumors (e.g. leiomyosarcomas, CRC, SCC) PTX3 expression was regulated epigenetically by methylation of the promoter region and of a putative enhancer. Thus, a crucial component of the humoral arm of innate immunity and regulator of Complement activation acts as an extrinsic oncosuppressor by dampening Complement-mediated, macrophage-sustained, tumor promoting inflammation.

Since previous studies demonstrated the protective role of PTX3 in *cancer-related inflammation* (CRI), in a murine model of mesenchymal cancer, we also addressed the role of PTX3 in murine model of epithelial origin, DMBA/TPA chemically induced skin cancer, focusing on other potential mechanisms causing CRI, in addition to the effect of macrophages and Complement.

The primary first experiments investigated PTX3 production throughout DMBA/TPA-induced skin carcinogenesis. During early carcinogenesis PTX3 was abundantly produced in the skin by infiltrating leukocytes, vessels and interstitial stroma. In late carcinogenesis, throughout chronic treatments with TPA, PTX3 was low and expressed mainly by the extracellular matrix.

PTX3-deficiency was associated with increased susceptibility to DMBA/TPA-induced skin cancer in terms of both papilloma number and incidence. Moreover, histological analysis and the expression profile of specific keratin proteins demonstrated a more aggressive behaviour of PTX3-deficient lesions, suggesting the protective role of PTX3 in both mesenchymal and epithelial murine models of cancer.

Further experiments were needed to characterize the molecular mechanisms, which explained the protective role of PTX3 in mouse skin carcinogenesis. PTX3 is a humoral pattern recognition molecule essential for resistance against selected pathogens including *A. fumigatus*[130], *P. aeruginosa*[135], uropathogenic *E. coli*[127]. PTX3 has antibody-like properties, such as recognition of microbial moieties[160], opsonization via Fcγ receptors[133], Complement activation and regulation of inflammation[148,161]. Indeed, it has long been known that $Ptx3^{-/-}$ mice display an exacerbated inflammation in diverse experimental models[155,156,157]. The enhanced inflammatory reactions observed in PTX3-deficient mice have been attributed to the regulation of Complement activation[150] or to the interaction with P-selectin mediated by PTX3[151]. Considering that tumor promoting inflammation is now recognized as an essential component of the tumor microenvironment[2,5], the increased susceptibility of PTX3-deficient mice to carcinogenesis is a reflection of its regulatory function on inflammation.

The biochemical and histological analysis of DMBA/TPA-treated skins showed the association between PTX3-deficiency and higher neutrophil infiltration and myeloperoxidase content in both early and late carcinogenesis. In addition, PTX3-deficiency was associated with higher level of the neutrophil-attractant CXCL-2 as well as pro-inflammatory mediators including IL-1α, IL-23. Both molecules are potent tumor-promoting cytokines[30,120]. Interestingly, $Ptx3^{-/-}$ skins showed high level of angiogenesis, consistent with a pro-angiogenic function of tumor-associated neutrophils[68].

It was therefore important to explain the relevance of neutrophils in the enhanced carcinogenesis of $Ptx3^{-/-}$ mice. The tumor-promoting functions of neutrophils are not without precedent and consist of reactive oxygen species-dependent and independent mechanisms[51,64]. The expression of the neutrophil chemokine receptor CXCR2 has been correlated to tumor progression. Short-term depletion of $Ly6G^{+}$ cells in established papillomas reduced their number and size[56]. Then, we used three different approaches, including P-selectin-deficient mice, a blocking antibody against Ly6G, and a genetic model of neutropenia, to address the role of neutrophils in skin carcinogenesis.

PTX3 has been shown to regulate P-selectin-dependent neutrophil recruitment in selected experimental models[151,157]. Accordingly, we interrogated the tumor susceptibility of P-selectin- and PTX3/P-selectin- double deficient mice in DMBA/TPA-induced skin carcinogenesis. P-$selectin^{-/-}$ mice were strongly protected. Interestingly, P-selectin deficiency reverted the phenotype of $Ptx3^{-/-}$ mice to the level of P-$selectin^{-/-}$ mice and reduced the neutrophil infiltration in both early and late carcinogenesis. Thus, these results suggest a mechanism of PTX3-mediated protection as a result of modulation of P-selectin-dependent neutrophil recruitment. However, the P-selectin pathway, as well as neutrophils, was not involved in the enhanced susceptibility to 3-MCA-carcinogenesis of Ptx3-deficient mice. These different results could be a consequence of the diversity of CRI in different organs or tumors[162].

The treatments with an antibodies direct against Ly6G throughout skin carcinogenesis reverted the phenotype of PTX3-deficient mice in terms of increased papilloma number. Thus, neutrophils represent the main actors for exacerbated inflammation in $Ptx3^{-/-}$ papilloma.

Finally, the use of $Csf3r^{-/-}$ mice, a genetic model of neutropenia, confirmed the relevance of neutrophil in CRI throughout mouse skin carcinogenesis.

PTX3 dampens inflammation favoring FH activity[148]. In 3-MCA induced fibrosarcoma $Ptx3^{-/-}$ lesions displayed higher C3 deposition than $Ptx3^{+/+}$, likely through the missing interaction of PTX3 with FH. Thereby, we focused on complement activation in the murine model of

DMBA/TPA-induced skin cancer. PTX3-deficiency was associated with increased cutaneous C3 levels in early carcinogenesis. To assess the role of complement in the phenotype of PTX3-deficient mice, we evaluated the tumor susceptibility of C3- deficient and PTX3/C3- double deficient mice in DMBA/TPA-induced skin cancer. Similarly to what observed in 3-MCA-carcinogenesis, C3-deficient mice are strongly protected against DMBA/TPA carcinogenesis. The results obtained here suggest that Complement is a key component of tumor-promoting cancer-related inflammation in both mesenchymal and epithelial cancers. In contrast to that observed in 3-MCA-carcinogenesis, in this preliminary experiment, C3-deficiency did not completely rescue the tumor susceptibility of $Ptx3^{-/-}$ mice in DMBA/TPA-induced late carcinogenesis suggesting that the exacerbated inflammation of $Ptx3^{-/-}$ papillomas is only partially dependent from the effect of Complement.

Antibodies have been shown to orchestrate CRI in a genetic model of epithelial carcinogenesis through FcγR-dependent mast cell and neutrophil recruitment[112]. PTX3 acts as a functional ancestor of antibodies and plays opsonic activity via FcγR, possibly competing to immunoglobulins[133]. PTX3-deficiency was associated with higher IgG1 deposition in both early and late carcinogenesis but currently we do not know whether this increase is responsible of higher inflammation observed in PTX3-deficient mice in chemically induced SCC.

Therefore, data derived by the investigation of three mechanisms, including the P-selectin-pathway, as well as the pharmacological and genetic approach to dampen neutrophil component, demonstrate the relevance of neutrophils for exacerbated inflammation of PTX3-deficient mice and their protumoral activity in chemically induced SCC.

The results obtained by our group, including my thesis work, suggest that a regulatory component of the humoral arm of innate immunity, PTX3, acts as an extrinsic oncosuppressor gene in mouse and human. Although the link between inflammation and cancer has long been

recognized[2,5], the role of humoral innate immunity and, in particular, the complement had never been investigated before.

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