Intracellular Osteopontin Controls the Release of TNF by Mast Cells to Restrain Neuroendocrine Prostate Cancer

Thesis

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Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.21954/ou.ro.00015856

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Intracellular osteopontin controls the release of TNFα by mast cells to restrain neuroendocrine prostate cancer

Thesis presented for the Degree of Doctor of Philosophy
The Open University, Milton Keynes (UK)

School of Life, Health, and Chemical Sciences

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M.Sc. in Molecular Biology
Personal Identifier: H5437238

October 2022
“Courage allows the successful woman to fail and learn powerful lessons from the failure. So that in the end, she didn’t fail at all.” – Maya Angelou
DECLARATION

The data presented in this thesis are original, were not previously used for any other PhD degree and were originated by me and by the cited below collaborators. During my PhD, I worked in the Molecular Immunology Unit, Department of Research, at the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan. My director of studies was Dr Elena Jachetti (PhD), and my supervisors were Dr Mario Paolo Colombo (PhD), and Prof Carlo Ennio Pucillo (PhD). Dr Jachetti and Dr Colombo are both from Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, and Prof Pucillo is head of the Immunology Section, Department of Medicine, University of Udine, Italy.

This work was supported by grants from the Italian Ministry of Health (GR-2016-02362484 to EJ), Cariplo Foundation (grant nr° 2018-0213 to EJ) and from Italian Association for Cancer Research (AIRC; Investigator Grant nr° 14194 to MPC).

I declare that all the in vitro and in vivo experiments were performed by myself, especially those regarding animal procedures (surgical castration, reconstitution with mast cells, sampling etc.).

The histopathological evaluation of murine prostates was performed by Prof Claudio Tripodo (MD, PhD) and Dr Valeria Cancila (PhD), Tumo Immunology Unit, Department of Health Sciences, University of Palermo, Italy.

Bioinformatic analyses were performed by Mr Matteo Milani and Ms Irene Tripodi bioinformaticians in my research group.

Some in vivo experiments were performed with the support of Mrs Laura Botti, a technician specialized in the manipulation and surgery of mouse models working in my research group.

Prostatectomies from prostate cancer patients undergoing neoadjuvant androgen deprivation therapy were kindly provided by Dr Giuseppe Renne (MD), Director of
Uropathology and Intraoperative Diagnostic, Istituto Europeo di Oncologia (IEO), Milan, Italy.

Prostatectomies from untreated prostate cancer patients were kindly provided by Dr Marco Bregni (MD), at the time he was working at the Oncology-Hematology Unit, ASST Valle Olona, Busto Arsizio, Italy.

MC/9 cells were kindly provided by Dr Barbara Frossi (PhD), Immunology Section, Department of Medicine, University of Udine, Italy.

MyD88−/− MCs were kindly provided by Prof Luigina Romani (PhD), Department of Medicine and Surgery, University of Perugia, Italy.

The multiplex immunoassay was performed with the help of Dr Francesca Bianchi (PhD), at the time she was working at the Molecular target Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori of Milan.

Confocal microscopy was performed with the help of Dr Sabina Sangaletti (PhD), working in my research group.

FACS sorting of MC/9 cells was performed at the sorting facility of the San Raffaele Scientific Institute, Milan, Italy.

Some experiments in vitro were repeated with the help of my supervisor Dr Elena Jachetti (PhD), Mrs Renata Ferri and Ms Irene Fischetti all working in my research group.

The results collected in this thesis are included in the following manuscript under preparation:


3
LIST OF ABBREVIATIONS

ADT= Androgen deprivation therapy
Ag= Antigen
AR= Androgen receptor
Arg1= Arginase
ARPI= Androgen receptor pathway inhibitor
BMP-6= Bone morphogenetic protein-6
BPH= Benign prostatic hyperplasia
CAFs= Cancer-associated fibroblasts
CgA= Chromogranin-A
CK= Cytokeratin
CPA= Carboxypeptidase A
CRPC= Castration-resistant prostate cancer
CSF-1= Colony stimulating factor-1
CTC= Circulating tumour cell
ctDNA= Circulating tumour DNA
ctRNA= Circulating tumour RNA
CZ= Central zone
ECM= Extracellular matrix
EV= Extracellular vesicle
EMT= Epithelial-mesenchymal transition
FACS= Fluorescence activated cell sorting
FFPE= Formalin-fixed paraffin-embedded
FGF= Fibroblast growth factor
FMO= Fluorescence minus one
FSP= Fibroblast-specific protein
GEP= Gene expression profile
GM-CSF= Granulocyte-macrophage colony-stimulating factor
GSEA= Gene set enrichment analysis
HGF= Hepatocyte growth factor
HGPIN= High-grade prostatic intraepithelial neoplasia
HIF-1α= Hypoxia-inducible factor-1 alpha
HMGB1= High mobility group protein B1
i.p.= Intraperitoneally
IFN= Interferon
IgE= Type E immunoglobulin
IL= Interleukin
IL-33R= Interleukin-33 receptor
iNOS= Inducible nitric oxide synthase
iOPN= Intracellular OPN
IRES= Internal ribosome entry site
ITAM= Immunoreceptor tyrosine-based activation motif
KIRs= Killer-Ig–like receptors
LH= Luteinizing hormone
LHRH= Luteinizing hormone-releasing hormone
LOXL2= Lysyl oxidase-like 2 receptor
LPS= Lipopolysaccharide
MAPK= Mitogen-activated protein kinase
MCc= Chymase MCs
MCP-1= Monocyte chemoattractant protein-1
MCs= Mast cells
M-CSF= Macrophage colony-stimulating factor
MCt= Tryptase MCs
MCtc=Tryptase and chymase MCs
MDSCs= Myeloid-derived suppressor cells
MFI= Mean fluorescent intensity
mMCP= Mouse mast cell protease
M-MDSCs= Monocytic myeloid-derived suppressor cells
MMPs= Metalloproteinases
MyD88= Myeloid differentiation primary-response gene 88
NCRs= Natural cytotoxicity receptors
NE= Neuroendocrine
NED= Neuroendocrine differentiation
NEPC= Neuroendocrine prostate cancer
NES= Normalized enrichment score
NFkB= nuclear factor kB
NK= Natural killer cells
NO= Nitric oxide
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron-specific enolase</td>
</tr>
<tr>
<td>ONECUT2</td>
<td>One cut homeobox 2</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OPNf</td>
<td>Full-length OPN</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Plk-1</td>
<td>Polo-like kinase-1</td>
</tr>
<tr>
<td>PMN-MDSCs</td>
<td>Polymorphonuclear myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PZ</td>
<td>Peripheral zone</td>
</tr>
<tr>
<td>REST</td>
<td>RE-1 silencing transcription factor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small-cell lung cancer</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
</tr>
<tr>
<td>SIBLINGs</td>
<td>Small integrin/binding ligand N-linked glycoproteins</td>
</tr>
<tr>
<td>sOPN</td>
<td>Secreted OPN</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and cysteine-rich</td>
</tr>
<tr>
<td>SV2A</td>
<td>Synaptic vesicle glycoprotein 2A</td>
</tr>
<tr>
<td>SYP</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumour-associated macrophages</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>t-NEPC</td>
<td>Treatment-related neuroendocrine prostate cancer</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T-cells</td>
</tr>
</tbody>
</table>
TRY = Tryptase
TZ = Transition zone
VEGF = Vascular endothelial growth factor
WT = Wild type
YAP1 = Yes-associated protein 1
α-SMA = Alpha-smooth muscle actin
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ABSTRACT

Fatal neuroendocrine prostate cancer (NEPC) often emerges in patients relapsing after hormone therapies. Besides, de-novo NEPC can rarely occur in treatment-naïve patients. The poor knowledge of mechanisms fostering NEPC growth hampers the development of effective therapies.

Investigating the tumour microenvironment, we found that mast cells (MCs) accumulate within hormone-sensitive prostate cancer favouring its growth, whereas are excluded by de-novo NEPC both in patients and in the TRAMP transgenic mouse model. Also, TRAMP mice backcrossed with MCs-deficient Kit<sup>Wsh</sup> mice showed increased frequency of de-novo NEPC. NEPC incidence similarly raised in TRAMP mice deficient for the matricellular protein osteopontin (OPN). Reconstituting Kit<sup>Wsh</sup>−TRAMP mice with wild type (WT), but not with OPN<sup>−/−</sup> MCs, lowered the frequency of NEPC to that of untreated TRAMP mice, suggesting that MCs exert their NEPC protecting function through OPN.

We found that both tumour-infiltrating and in-vitro cultured MCs stain positive for OPN but release a tiny amount of OPN in supernatants if compared to NEPC cells. Notably, OPN has both secreted (sOPN) and intracellular (iOPN) forms; the latter can bind to MyD88 and regulate the signalling downstream toll-like receptors (TLRs). We then hypothesized that iOPN in MCs can control the TLR-dependent release of antiproliferative cytokines.

Indeed, WT, but not OPN<sup>−/−</sup> or MyD88<sup>−/−</sup>, MCs inhibited the proliferation of NEPC cells in vitro. This effect was mediated by tumor necrosis factor alpha (TNFα), specifically secreted by MCs after the contact with NEPC cells. We confirmed that MCs infiltrating both murine and human incipient NEPC lesions express TNFα. Moreover, the reconstitution with TNFα<sup>−/−</sup> MCs did not reduce the abnormal frequency of NEPC in Kit<sup>Wsh</sup>-TRAMP mice. In human and murine data sets of overt NEPC, genes related to TLRs signalling, including TNFα were downregulated.

Our data indicate that TLRs/MyD88/iOPN-mediated pathways induce MCs to release TNFα to restrain NEPC, suggesting investigating MC-based approaches for NEPC.
INTRODUCTION

1. PROSTATE CANCER

1.1 Anatomy and histology of human prostate

The human prostate is a little gland, part of the male reproductive organ, located below the urinary bladder and in front of the rectum (1). The main function of the prostate is to produce and secrete the seminal fluid that, together with fluids produced by seminal vesicles, compose the semen (2). One of the main proteins in the prostatic fluid is prostate-specific antigen (PSA), whose levels increase in prostate cancer (PCa). For this reason, PSA is used as a biomarker for the detection of PCa (3).

The anatomy of the prostate was described by McNeal as constituted by 5 lobes (anterior, posterior, median, right lateral and left lateral) and divided into 3 zones identified and characterized as the transition zone, the central zone, and the peripheral zone [(4) and Figure 1], with different features and a tendency to develop benign or malignant neoplasms.

Fig. 1 Prostate zone anatomy. Created in BioRender.com
The peripheral zone (PZ) represents about 70% of the normal prostate and is the main site of origin of cancers. The transition zone (TZ) constitutes 5% of the prostate volume and is more susceptible to developing benign prostatic hyperplasia. The central zone (CZ) surrounds the TZ and represents 25% of the prostate volume. The CZ is not commonly the site of tumour onset but could be secondarily implicated by prostate cancer arising in other zones.

The epithelium of the prostate is formed by acini and ducts that consist of 2 main cell types: luminal and basal cells ([5] and figure 2).

**Fig. 2 Schematic cell type in the prostate. Created in BioRender.com**

Luminal cells are secretory cells that contribute to the formation of the seminal fluid and express cytokeratin (CK) 8, CK18 and, androgen receptor (AR). Basal cells are located beneath the luminal epithelium, lack secretory activity, and express p63, CK5, and CK14 with low or undetectable AR levels (6). Intermingled between basal cells, neuroendocrine (NE) cells are rarely found. These cells secrete neuropeptides and other soluble factors like hormones, serotonin, and calcitonin that promote homeostasis and growth (7). Furthermore, NE cells express specific neuronal markers such as synaptophysin (SYP), Chromogranin-A (CgA), and neuron-specific enolase (NSE) (8).

The stromal compartment of the prostate is constituted by the extracellular matrix (ECM) and is populated by several cells such as fibroblasts and smooth muscle cells fused within a pseudo capsule with an outer layer of collagen fibres (9,10) as well as various type of immune cells. The prostate is dependent on testicular androgens to
guarantee epithelial cell maintenance, differentiation, and function mediated mainly by AR (11). Epithelial and stromal cells react to testosterone in a mutual and bidirectional manner to assure prostate architecture and homeostasis (12,13). Disruption of the balance between these 2 cellular compartments but also the deregulation of the AR pathway can be detrimental and can lead to the onset of PCa.

1.2 Prostate cancer

Ageing men can develop several pathological diseases of the prostate extending from inflammation to benign prostatic hyperplasia (BPH) and malignant PCa. However, recent observations suggest that PCa diagnoses have increased globally with a rate averaging 2% per year in adolescents and young adults, defined as ages 15-39 inclusive. These patients display mostly aggressive disease and have worse survival than middle-aged and old men (14). BPH is a condition that represents an abnormal enlargement of prostate volume; it is not a pre-malignant condition and is commonly treated with different drugs depending on patients’ symptom burden (15,16).

Prostatitis is the most common inflammation in men, classified into 4 different categories (acute bacterial prostatitis, chronic bacterial prostatitis, chronic pelvic pain syndrome, and asymptomatic prostatitis) and the choice of the treatment is related to the different types (17). Both BPH and prostatitis represent risk factors for PCa, together with obesity, alcohol consumption, and sexual activity (18).

A precursor of PCa is high-grade prostate intraepithelial neoplasia (HGPIN). HGPIN is described as an abnormal proliferation of the glandular architecture that occurs especially in the peripheral zone of the prostate (75-80%), while it is rarely found in the transition zone (10-15%) and in the central zone (5%) (19,20). It can be detected only by biopsy due to the low levels of PSA detected in circulation in affected patients. HGPIN shares with PCa some genetic features and molecular markers, confirming that HGPIN represents an intermediate stage between benign epithelium and neoplasia. Furthermore, it is well known that most patients with HGPIN develop carcinoma within 10 years (19,21).

PCa is the most common cancer diagnosed in men with about 1.250.000 new cases every year worldwide and it is the second leading cancer-related death among men after lung cancer ((22,23) and Figure 3). The survival from PCa improves every year due to enhanced screening and detection of low-grade disease with PSA testing and
to innovations in characterization and treatments (22). However, over-diagnosis associated with PSA screening is a critical issue in PCa that can lead to overtreatment, even with a slight impact on mortality (24).

Some evidence shows that reducing screenings in men over 60 to those with PSA above the median (>1 ng/ml) and screening men over 70 in selected conditions could decrease overdiagnosis (25). PCa is a heterogeneous disease with slow progression. In most cases PCa turns out to be indolent, thus bypassing the impact on long-term survival and patients’ quality of life (26). Generally, following the detection of an increased level of PSA, the diagnosis of PCa is confirmed with a biopsy and histologic evaluation. The Gleason Score is the primary method to grade PCa and is based on the glandular architecture and differentiation of the tumour. The Gleason Score ranges from 1 to 5, with the less glandular the microscopic appearance corresponding to the higher grade. Since PCa is a multifocal tumour in which different areas within the same prostate can have different grades of differentiation, the Gleason Score is the sum of 2 values in which the first represents the grade of the predominant pattern of the tumour and the second the common pattern adjacent to

---

**Fig. 3 Ten Leading Cancer Types for the Estimated New Cancer Cases and deaths by Sex in United States (2022).**

*Taken from the website of the American Cancer Society, (Cancer Facts and Statistics 2022), upon receiving proper authorization to utilize the picture in this thesis.*
the main area. Thus, the total Gleason Score ranges from 2 to 10, in which ≤ 6 indicates indolent cancer whereas scores of 8 or more are related to poorly differentiated tumours with dismal prognosis (27). The Gleason Score method was introduced in 1974 by Donald Gleason and the Veterans Administration Cooperative Urologic Research Group (28) but it has been revised firstly in 2005 (29) and then in 2014 (30) by the International Society of Urological Pathology (Figure 4). This update was necessary since biopsy and radical prostatectomy usually are discordant in the assignment of Gleason Score, mainly because the multifocal features of the tumour are not well represented in the biopsy.

![Gleason Score Diagram](image)

**Fig. 4 Gleason score from 1992 to 2014.**
*Taken from Sunassee et al., Intraductal carcinoma of prostate, grade group, and molecular pathology: recent advances and practical implication, Ann Urol Oncol 2019, upon receiving proper authorization to utilize the picture in this thesis.*

The review of the Gleason Score involves a 5-grade system with grade 1 (Gleason Score ≤ 6), grade 2 (Gleason Score = 3+4), grade 3 (Gleason score = 4+3), grade 4 (Gleason Score = 4+4, 3+5 or 5+3), and grade 5 (Gleason Score = 9 or 10). The new classification resolves the issue of the great differences of tumours scored as 7 that can be the result either of a Gleason Score of 3+4 or 4+3, which have remarkably different risk grades and prognostic impact.

Truly, this system categorises tumours with Gleason Score = 3+4 (Group Grade 2) as low risk to be followed by active surveillance, whereas tumours with Gleason Score = 4+3 (Group Grade 3) are high risk in which further therapeutic approach is necessary (30).
Like other types of cancers, PCa can be also classified by the TNM system in which T is referred to the size and the extension of the main tumour area, N represents the number of metastases in regional lymph nodes, and M indicates the presence of distant metastases (31). PCa can be also classified as localized, locally advanced, and advanced (metastatic). Localized PCa remains confined to the organ. In turn, localized PCa can be divided into 3 risk groups: low-risk, intermediate-risk, and high-risk depending on the size of the tumour (T stage), the pattern that cancer cells present when looked at microscope (Gleason score), and PSA levels.

Locally advanced PCa is a condition in which cancer cells can invade other sites nearby the prostate (bladder, seminal vesicles, rectum, and lymph nodes close to the prostate gland) (32). Advanced PCa is the condition in which cancer cells broadly metastasize to other parts of the body, especially in distant lymph nodes and bones (6).

The most common neoplasia of the prostate is acinar-type adenocarcinoma (95%) that originates from luminal cells with secretory properties. These tumours express AR and are negative for basal markers (6,33). Other histologic subtypes of PCa include basal cell and adenoid cystic carcinoma, primary squamous carcinoma, primary urothelial carcinoma, and primary sarcomatoid carcinoma (33). As better described in the following paragraphs, neuroendocrine tumours (NEPC) can also arise in the prostate, either de-novo or as a mechanism of resistance to hormone therapies.

1.3 Therapeutic approaches and mechanisms of resistance

PCa treatment depends on the different features that characterize the tumour and the clinical history of the patients. These include the age of the patient, and the pathological features and the grade of the tumour but also the presence of comorbidities that can influence the response to therapy. Therapeutic approaches for PCa involve active surveillance, watchful waiting, radiotherapy, chemotherapy, surgery, immunotherapy, and hormone therapy.

Active surveillance is a method that reduces the overtreatment of patients. It consists of a follow-up every 6 months with a PSA test accompanied by a digital rectal exam (34), while watchful waiting is an observation methodology with less frequent
follow-up than active surveillance (35). These procedures are mainly used for patients who have low risk localized PCa (36).

**Surgery** options include open surgery, laparoscopic, and robot-assisted laparoscopic radical prostatectomy. The experience and clinical decisions of the surgeon are more important rather than the procedure chosen to determine the outcome (37).

**Radiotherapy** for PCa involves external beam radiotherapy or brachytherapy. The first one consists of beams derived from an external source while brachytherapy uses little radioactive elements placed directly into the prostate (38). Radiotherapy is usually administered as adjuvant therapy after radical prostatectomy or in combination with androgen deprivation therapy (ADT) in locally advanced PCa patients (39).

**Chemotherapeutic drugs** typically used for PCa are Docetaxel, Cabazitaxel, Mitoxantrone, and Estramustine. This therapeutic regimen is typically used in advanced prostate cancer, especially in castration-resistant (CRPC) and hormone-insensitive PCa (40).

**Immunotherapy** involves vaccine-based therapies such as sipuleucel-T and PROSTVAC or immune checkpoint inhibitors. Sipuleucel-T was the first FDA-approved therapeutic cancer vaccine. It is autologous cellular immunotherapy based on the collection of a patient’s dendritic cells that are manipulated *in vitro* to express a fusion protein of granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP) and then reinfused into the patient (41). However, despite a prolonged survival in asymptomatic or slightly symptomatic metastatic CRPC patients, sipuleucel-T did not significantly affect progression-free survival (42). Furthermore, it was withdrawn in 2015 at the request of the manufacturer because no further European development was planned (43). PROSTVAC is another vaccine-based therapy that exploits recombinant poxviruses expressing PSA and 3 T-cell costimulatory molecules (B7-1, ICAM-1, and LFA-3) to enhance T-cell response. After injection in patients, recombinant poxviruses infect dendritic cells, which in turn, present recombinant proteins on their surface and, after interaction with T-cells, lead to a targeted immune response (44). However, also PROSTVAC did not show improvement in survival in metastatic CRPC patients so far (45).

Immune checkpoints are molecules expressed on the surface of tumour or regulatory immune cells, able to down regulate T-cell response when engaging the cognate
receptor. The most important immune checkpoints are CTLA-4 and PD-1, and monoclonal antibodies inhibiting their function are now commonly used for the therapy of many solid tumours including melanoma and lung cancer (46). However, immune checkpoint inhibitor monotherapy showed poor effectiveness in metastatic CRPC likely because of the lack of effector T-cells within the tumour microenvironment. Although being effective only in a few PCa patients, immunotherapy remains a promising area of research for metastatic CRPC worth to be further investigated (45).

**Hormone therapy** includes different strategies aiming to reduce the levels of male hormones or blunt their signalling pathway.

Orchiectomy is a form of surgery based on the removal of testicles that are the main source of androgens. This represents a permanent method, but many patients are not willing to undergo this procedure, opting for chemical castration approaches. Chemical castration is obtained by administration of luteinizing hormone-releasing hormone (LHRH) agonists such as leuprolelin, triptorelin, and goserelin that block the production of luteinizing hormone (LH) and testosterone by the pituitary gland and testicles, thus reducing the level of circulating androgen. LHRH agonists have the same effect as orchiectomy in terms of the survival of patients after the therapy (47).

Antiandrogen drugs, also called AR pathway inhibitors (ARPI), involve compounds that block the binding between androgens and AR on tumour cells or the activity of AR itself, resulting in inhibition of cancer growth. First-generation nonsteroidal antiandrogens (bicalutamide, flutamide, and nilutamide) act selectively against AR and, among them, bicalutamide was considered the most efficient in terms of toxicity and safety. Unfortunately, these therapies do not completely abrogate AR activity and second-generation ARPI have been developed, including abiraterone acetate, enzalutamide, and the recently approved apalutamide and darolutamide (48,49).

Despite the initial response, most of PCa switch to CRPC as resistance mechanism to hormone therapy. Mechanisms of resistance include amplification of AR, or alternative splicing to generate constitutively active forms of the receptor (ARV7) (50). An additional resistance mechanism is linked to neuroendocrine differentiation (NED) and the emergence of a tumour variant completely independent from the androgens, identified as NEPC (51).
1.4 NEPC

NEPC is the most aggressive phenotype of PCa and represents the late stage of the disease. It can arise as treatment-related NEPC (t-NEPC) in patients developing resistance to hormone therapy or as de-novo in untreated patients. Although de-novo NEPC represents a rare tumour (about 1% of patients at diagnosis), t-NEPC is gaining clinical importance in recent years. Indeed, recent evidence suggests that nearly 20% of patients with progressive metastatic CRPC have NEPC (52). Diagnosis of NEPC is very difficult due to its emergence in patients with multiple metastases, which disheartens clinicians to perform biopsies. As a result, the incidence of NEPC is usually underestimated and some patients with NEPC are treated with an inappropriate regimen suitable for AR-positive adenocarcinoma PCa, with no achievement. Furthermore, given the foreseen clinical use of ADT/ARPI in neo-adjuvant settings in high-risk PCa or metastatic CRPC patients, now being tested in clinical trials (NCT04356430 or NCT00887198, respectively), the incidence of NEPC is expected to rise in the coming years.

Patients with this extremely aggressive subtype of PCa have very limited therapeutic options, and the overall survival is approximately 1 year from the time of the diagnosis (53). Since NEPC shares histology features with small-cell lung cancer (SCLC), patients are usually treated with platinum-based therapy. Unfortunately, most patients progress rapidly and there is no second-line treatment (54,55). Thus, there is an important need to implement diagnostic biomarkers and to set up new therapies for NEPC.

The development of suitable biomarkers for NEPC diagnosis is a critical issue in the Italian Healthcare System, as biopsies for follow up of relapsing/metastatic disease are not routinely made in prostate cancer patients (AIOM guidelines). In this scenario, circulating biomarkers could represent a great opportunity to follow the adenocarcinoma-neuroendocrine transition, and to guide therapeutic decisions. Liquid biopsies rely on circulating tumour cells (CTCs), extracellular vesicles (EVs), circulating tumour DNA (ctDNA) or RNA (ctRNA) (56). Currently, CTCs seems to have a potential prognostic function in NEPC. Beltran and colleagues have demonstrated that CTCs from NEPC patients have peculiar distinct properties rather than CTCs from CRPC patients. In particular, NEPC-CTCs are smaller than CRPC-CTCs, express low AR and cytokeratin, and present abnormal nuclear and
cytoplasmic features. Furthermore, NEPC-CTCs were present in about 10% of CRPC patients with aggressive features, suggesting that these CTCs may provide useful information to monitor patients who undergone resistance to therapy and disease progression (57).

t-NEPC and de-novo NEPC have common genomic alterations (e.g., TMPRSS2-ERG fusion) and present the same transcriptomic profile, although de-novo NEPC has not had any AR alteration as in ADT-exposed CRPC (58). This suggests that, regardless of their origin, t-NEPC and de-novo NEPC take advantage of common pathways that can be exploited for therapeutic approaches.

The origin of NEPC is still debated since it is not clear whether it arises from trans-differentiation of pre-existing adenocarcinoma cells, caused by the pressure of ADT/ARPI, or by a different population of basal/NE cells. Nevertheless, the first theory is now well accepted, and several data are supporting that lineage plasticity can drive NEPC differentiation from adenocarcinoma (55,59).

Histologically NEPC is characterized by small-cell morphology, expression of NE markers like SYP and CgA, loss of AR expression and/or activity, total loss of the glandular architecture of the prostate, and high proliferation rate and metastatic potential (60,61). However, in some cases, NEPC can have mixed features, with large-size tumour cells expressing AR and its related genes as well as NE markers (hybrid tumours). These can represent transition steps in the plasticity process that drives the differentiation of adenocarcinoma towards NEPC (62). Indeed, the loss of RB1 and PTEN, the mutation or deletion of TP53, the co-amplification of MYCN and AURKA, and the downregulation of transcription factors (e.g., REST) lead to the reprogramming of the adenocarcinoma PCa to NEPC, indicating a key role for lineage plasticity in NEPC development (55,63–65). Loss of both RB1 and TP53 increases the expression of the epigenetic regulators SOX2 and EZH2 that, in turn, guide lineage plasticity toward AR-independence, resistance to treatments, and NEPC onset (66,67). Dardenne and colleagues also demonstrated that N-MYC and its stabilization by AURKA and AKT1 induces EZH2-mediated repression of AR signalling and drives a molecular program that culminates in NEPC differentiation (68). Besides adding cues to the trans-differentiation theory, these studies highlight also that EZH2, SOX2, and AURKA inhibitors could be effective strategies against NEPC, aimed to restore the sensitivity to ADT/ARPI. Nevertheless, Alisertib, an AURKA inhibitor, was evaluated in a phase II clinical trial (NCT01799278) in NEPC
patients. Even though the study did not reach the primary end of 6-month radiographic progression free survival, some patients with features of Aurora-A and N-myc activation gave clinical benefit from the treatment with resolution of liver metastases and prolonged stable disease (69). Furthermore, exploiting similarities between NEPC and SCLC, Puca and colleagues recently discovered the DLL3 protein as a novel potential therapeutic target for NEPC (70). Interestingly, DLL3 inhibitors are now tested in clinical trials for both NEPC and SCLC (NCT04471727, NCT04702737) (71). These new potential therapeutic options pave the way for improved treatments for NEPC, which is still a fatal tumour. The results of the aforementioned clinical trials could be exploited for new therapeutic interventions also in combination with platinum-based regimens, that is one of the treatments usually adopted in NEPC patients.
2. THE TUMOUR MICROENVIRONMENT

2.1 Stroma and immune cells among tumour microenvironment

Cancer development and progression are influenced by the interaction between cancer cells and the tumour microenvironment (TME), whose components actively promote progression, metastatic process, and therapeutic resistance. TME comprises different classes of cells, including mesenchymal cells, fibroblasts, endothelial cells, and immune cells as well as non-cellular components represented by ECM proteins (Figure 5).

![Fig. 5 Schematic illustration of TME composition. Created in BioRender.com](image)

Cancer-associated fibroblasts (CAFs) are one of the main components of the TME in many solid tumours. These cells derive from stromal fibroblasts differentiated into activated phenotype upon tumour-derived stimuli. Their perpetual activation allows neither reverting to normal phenotype nor undergoing apoptosis (72). CAFs resemble myofibroblasts involved in wound-healing, but they differ from normal fibroblasts in their increased expression of alpha-smooth muscle actin (α-SMA), vimentin, fibroblast-specific protein (FSP), platelet-derived growth factor (PDGF) and decreased expression of desmin (73). CAFs are the major source of metalloproteinases (MMPs), transforming growth factor-beta (TGF-β), interleukin (IL)-6, fibroblast growth factor (FGF), hypoxia-inducible factor-1 alpha (HIF-1α),
and vascular endothelial growth factor (VEGF), all produced as a result of interaction with cancer cells, and which stimulates tumour growth and invasiveness (74,75).

CAFs are present in almost all solid tumours modulating and influencing other cells within the TME in a tumour-promoting or tumour-inhibiting manner. Moreover, the excessive deposition of ECM together with the production of MMPs by CAFs promote biochemical changes in the matrix which guides tumour growth and invasiveness, contributing also to the metastatic process (76). For instance, CAFs can organize fibronectin in aligned fibres that facilitate the migration of PCa cells (77).

In pancreatic cancer, CAFs secrete cytokines and chemokines such as IL-6, VEGF, macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1), and stromal cell-derived factor-1 (SDF-1) that promote the differentiation of tumour associated macrophages (TAMs) into pro-tumorigenic (M2) phenotype leads to an immunosuppressive environment (78,79). It has been described that CAFs stimulate angiogenesis through the release of SDF-1 and enhance breast cancer growth by paracrine stimulation via CXCR4 expressed by breast carcinoma cells (80). In colorectal cancer, CAFs can secrete hepatocyte growth factor (HGF) to trigger both mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathway in cancer cells, thus assuring their survival and invasion (81). In head and neck squamous cell carcinoma CAFs help cancer cells invasion through the degradation of ECM, especially collagen (82).

In PCa, CAFs can guide invasiveness and progression of low tumorigenic adenocarcinoma cells towards CRPC and bone metastases (83). It has been described that CAF-derived factors have a key role in inducing genomic methylation changes leading to epithelial-mesenchymal transition (EMT), stemness, and growth of PC3 and DU145 cells (84). Indeed, the role of CAFs in inducing EMT has been demonstrated also in lung cancer in which CAFs can activate the IL6/STAT3 signalling pathway activating EMT and enhancing the metastasis cascade in vitro and in vivo (85). Notably, in PCa IL-6 derived from cancer cells is important for CAF activation and release of MMPs which, in turn, promote EMT, tumour aggressiveness and metastases (86). CAFs also induce upregulation of steroid and cholesterol biosynthesis and metabolism in PCa cells that promote ADT resistance and consequently tumour growth (87). Nguyen and co-workers discovered that the lysyl oxidase-like 2 receptor (LOXL2) has a key role in intracellular communication
between PCa cells and CAFs also promoting CAF motility, organization of ECM and increase of tumour cell migration (88).

Besides CAFs, different immune cell types can populate the TME. Tumour infiltrating immune cells can either directly attack cancer cells or inhibiting immune recognition to promote cancer growth. To explain the complex interactions between the tumour and the immune system, the concept of cancer immunoediting was proposed (89). This process is divided into 3 stages, called the 3 “Es”: elimination, equilibrium, and escape. In the early stage of cancer development, immune cells in the TME have the potential to attack tumour cells trying to impede neoplastic progression (elimination). At this stage, most cancer cells are eliminated through the intervention of innate and adaptive immunity. However, survivor cancer cells acquire a non-immunogenic phenotype, evading immune recognition (equilibrium). Then, tumour acquires the ability to instruct immune and stromal cells to enhance and maintain tumour proliferation and metastatic spread through the secretion of several pro-tumorigenic and pro-angiogenic factors. The last stage (escape) occurs when the immune system ultimately fails to destroy the tumour. In this scenario cancer cell variants can grow exploiting several mechanisms of resistance to immune detection, allowing the tumour to become clinically detectable (90,91).

The major immune cell component of the TME are TAMs, which can directly interact with cancer cells or with other cells of the TME promoting immunosuppression, angiogenesis, and metastasis (92). TAMs can be distinguished in 2 different polarization states: classically activated “M1” macrophages with tumour-suppression properties or alternatively activated “M2” macrophages that promote tumour growth (93). TAMs are a population in a state of constant transition between M1 and M2 polarization stages, and the balance of each form is controlled by different signals that they receive from TME (94).

In PCa patients, the high presence of TAMs correlates with poor prognosis and their M2 polarization is associated with tumour extension (95). Di Mitri and colleagues demonstrated that in PCa TAMs express high levels of CXCR2 and that the use of CXCR2 antagonist promotes the re-education of TAMs towards a TNFα-releasing phenotype, leading to cancer inhibition (96). Moreover, a different study showed that the inhibition of colony stimulator factor-1 (CSF-1) in combination with ADT
decreases TAM recruitment blocking cancer progression and promoting a more durable response to ADT monotherapy (97). Together with TAM, myeloid-derived suppressor cells (MDSCs) represent a significant portion of immune populations infiltrating the TME, endowed with protumoral properties. In mice these cells are classified into 2 subtypes: polymorphonuclear MDSCs (PMN-MDSCs: CD11b+ Ly6G+ Ly6Chq) and monocytic MDSCs (M-MDSCs: CD11b+ Ly6G+ Ly6Chhi). In humans, both types express CD11b and the general myeloid marker CD33 but M-MDSCs express CD14 while PMN-MDSCs express CD15 and CD66b. Furthermore, it is possible to distinguish MDSCs from monocytes by their absence of HLA-DR. M-MDSCs are also commonly characterized by their high production of nitric oxide (NO) and expression of inducible NO synthase (iNOS) whereas PMN-MDSCs produce high levels of reactive oxygen species (ROS) (98).

The increased level of MDSCs in bone marrow, spleen, and blood of cancer patients or cancer-bearing mice is associated with cancer development and progression (99). In PCa PMN-MDSCs are the most relevant subtype (100,101). It has been found that PMN-MDSCs are accumulated in the blood and prostates of PCa patients and that their number increases from localized to metastatic disease. It has been described that the epithelium-specific Ptten deletion in the prostate promotes the secretion of CSF-1 and IL-1β with consequent expansion of MDSCs (102). Furthermore, tumours derived from PTEN-null mice present high levels of PMN-MDSCs that protect cancer cells from senescence supporting the tumour growth (103).

Wang and colleagues demonstrated that yes-associated protein 1 (YAP1) is involved in the recruitment of MDSCs through the transcription of Cxcl5 (104). Moreover, in PCa also IL-6 and IL-8 could be responsible for MDSCs accumulation, which increases with the stage of cancer and correlates with poor prognosis (105). Furthermore, Hossain and colleagues, demonstrated that the silencing of STAT3 in PMN-MDSCs reduced the production of arginase 1 (Arg1) blocking their immunosuppressive effects on CD8+ T-cells (106). My laboratory also discovered that the interaction with mast cells (MCs) via CD40/CD40L leads to upregulation of Arg1, iNOS, and STAT3 by PMN-MDSCs enhancing their immunosuppressive function toward CD8+ T-cells and prostate cancer growth (100). Finally, it has been shown that PMN-MDCs accumulate in prostates after ADT and promote castration resistance through secretion of IL-23 that in turn activates AR signalling in tumour
cells (107). Among myeloid cells, also MCs can be frequently found in the TME of different solid tumours. Being the object of this thesis, their biology and functions will be further discussed in a dedicated chapter.

Across multiple cancers, the presence of infiltrating T lymphocytes has been linked to a good prognosis. The concept of the immunoscore based on the enumeration of 2 lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) counted in tumour centre or invasive margin (108) and the development of different tumour inflammation signatures led to the definition of inflamed/ “hot” tumours versus non-inflamed/ “cold” tumours (109,110).

Even though in many solid tumours the presence of a high number of infiltrating CD8$^+$ T-cells is associated with a good prognosis (111), in PCa contradictory papers suggest that in the prostate it is associated with poor prognosis and disease progression (112,113). Therefore, PCa is considered a “cold” tumour. This is because CD8$^+$ T-cells in most PCa patients are exhausted and dysfunctional due to immunosuppressive mechanisms (114). One of these immunosuppressive mechanisms is conducted by CD4$^+$ regulatory T-cells (Tregs) that can suppress anti-tumour CD8$^+$ T-cells response to promote cancer growth (115,116). Moreover, a high number of tumour-infiltrating CD4$^+$ Tregs is associated with lethal PCa (117). In addition to this classical subset of Tregs (CD4$^+$ CD25$^+$ FoxP3$^+$ T-cells), also a non-conventional subset of suppressive CD8$^+$ FoxP3$^+$ T-cells has been described in PCa (118).

Cancer-infiltrating natural killer (NK) cells have acquired importance in the cancer field since they are involved in anti-tumour mechanisms. These cells are part of the innate immunity with robust cytotoxic activity against different targets such as virus-infected cells, stressed cells, and tumour cells (119). NK cells lack T or B receptors but present several receptors such as killer-Ig–like receptors (KIRs), natural cytotoxicity receptors (NCRs), TNF-related apoptosis-inducing ligand (TRAIL), NKG2D, as well as several cytokines and chemokines receptors (i.e., IL-2, IL-15, CXCR4, CXCR3 etc.) that permit NK cells to engage different targets (120). It has been found that NK cell presence correlates with prognosis in several tumours, including gastric carcinoma, melanoma, lymphoma, and leukaemia (121–125).

A high number of human prostate infiltrating NK cells has been associated with a lower risk of progression suggesting that NK cells can have a protective role towards PCa (126). However, a study of Pasero and co-workers described that NK cells
infiltrating PCa have low cytotoxic ability due to the secretion of TGF-β by tumour cells, which suppress NK cells functions. In addition, they found that in co-culture experiments PCa cells induced the expression of inhibitory receptor ILT2 and the downregulation of stimulatory receptors NKp46, NKG2D, and CD16 on NK cells, thus blocking their activation when encountering tumour cells (127). Additionally, Guerra and colleagues unravelled that more aggressive tumours occur in the NKG2D-deficient transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (128). Gallazzi and collaborators also described that NK cells isolated from a PCa patient have acquired pro-angiogenic and pro-inflammatory activity with downregulation of NKG2D receptor and less degranulation capability (129).

These pieces of evidence emphasize that the continuous crosstalk between cancer cells and TME may play a key role in treatment resistance and in mediating or counteracting tumour progression. Further studies are required to better understand the interplay between tumour cells and the different cells residing in prostate TME that can influence cancer behaviour.

### 2.2 ECM

ECM is the major constituent of the TME, providing structural support, but also being involved in maintaining homeostasis, differentiation, and growth of the cells. ECM comprises distinct components including glycoproteins, proteoglycans, polysaccharides, and fibrous proteins that are implicated both in physiological and pathological conditions and are divided in 2 categories: structural and non-structural proteins. The structural proteins constitute the support of the cells and play a role in cell adhesion and migration. Examples of these proteins are collagens, elastin, fibronectin, and laminin (130). On the contrary, non-structural proteins include matricellular proteins such as thrombospondin, osteopontin (OPN), secreted protein acidic and cysteine-rich (SPARC), and tenascin-C that interact with cell-surface receptors, proteases, hormones, and other proteins, modulating cell functions (131). Every normal tissue contains its heterogeneous ECM that is biochemically different in composition and stiffness compared to cancer-associated ECM. However, changes in ECM properties have been linked to cancer development and evolution, and in this context matricellular proteins have a regulatory function in bridging inflammation and cancer. Indeed, matricellular protein can be abnormally expressed by both cancer
and surrounding stroma and immune cells (132). Indeed, ECM can be both a sensor and a source of signals that influence the mutual relationship between tumour and stromal cells (133,134).

2.2.1 OPN

The matricellular protein OPN is an O-glycosylated phosphoprotein member of the small integrin/binding ligand N/linked glycoproteins (SIBLINGs) family. It is encoded by the Spp1 gene located in chromosome 4 in humans and chromosome 5 in mice. OPN is a 33 kDa protein, composed of 314 amino acids in humans and 297 in mice. Following posttranslational modifications, such as O-linked glycosylation, tyrosine sulfation as well as Ser/Thr phosphorylation, its molecular weight increases to about 44 kDa. OPN is produced by different kinds of cells, like macrophages, neutrophils, dendritic cells, NK cells, T- and B- lymphocytes, MCs as well as epithelial cells and fibroblasts (135,136).

The sequence of full-length OPN (OPNf) contains an aspartate-rich region, 2 heparin-binding sites, proteolytic cleavage sites for thrombin and metalloproteinases and, located nearby the C-terminal portion, a region of interaction with CD44 receptor and its variants (CD44v3, CD44v6, and CD44v7). The thrombin-mediated cleavage of OPN permits the exposure of a cryptic integrin-binding site (SVVYGLR in humans, SLAYGLR in mice). The N-terminal thrombin fragment has been described to promote adhesion, spreading, and migration of cells, especially leukocytes, expressing α4β7 and α9β1 integrins (135). Also, OPN contains an arginine-glycine-aspartate (RGD) domain that is shared by several matricellular proteins and is important for the binding with many integrins, including αvβ1, αvβ3, αvβ5, αvβ6, and α5β1 (137). Notably, the SPP1 gene consists of seven exons that can be alternatively spliced to produce new variants of the protein. OPN-a is considered to be the canonical transcript of OPN coding for OPNf, comprising all the exons of the gene. OPN-b and OPN-c are 2 splice variants that lack exon 5 and exon 4 respectively. These splicing variants occur at the N-terminal domain of OPN protein, but little information is available about this region (138). What is known is that OPN-b lacks a sequence containing one of the clusters of phosphorylated serine and threonine residues (139) while OPN-c misses glutamine residues in which
transglutaminase cross-linking occurs (140). The role of these splicing variants has been investigated mostly in cancer.

Furthermore, studies in mice revealed that OPNf exists in a secreted (sOPN) or intracellular (iOPN) form. The iOPN does not derive from alternative splicing of the Spp1 gene but it is the result of an alternative translation site downstream of the canonical AUG codon of the same mRNA (OPNf). iOPN differs from sOPN because it lacks the N-terminal signal sequence needed for its secretion in the extracellular space ((141) and figure 6).

sOPN can be secreted by tumour and immune or non-immune stromal cells. It acts like a cytokine and is involved in many physiological functions, including cell adhesion, inflammation, migration, survival, and apoptosis. These biological roles are due to its capacity to interact with several surface receptors on different cells (142).

![Fig. 6 Alternative translation of Opn mRNA that generates iOPN isoform. Created in BioRender.com](image)

iOPN is prevalently expressed by myeloid cells and is mainly located in the cytoplasm (143) where it works like an adaptor protein rather than as a catalytic molecule. Indeed, iOPN has been described to co-localize with myeloid differentiation primary-response gene 88 (MyD88) downstream of most of the toll-like receptors (TLRs) such as TLR9, TLR2 and TLR4 (144–146). In this scenario, the role of iOPN is dichotomous. iOPN was found to promote TLR9-mediated interferon (IFN)-α production in plasmacytoid dendritic cells (144). On the contrary,
it can strongly suppress the TLR4-mediated production of pro-inflammatory cytokines such as IL-6, IL-12, and TNFα in macrophages (145,147). It has also been found that iOPN can interact with TLR2, dectin-1 and mannose receptors to activate anti-fungal innate immunity (148). Furthermore, iOPN is a component of a CD44-ezrin/radixin/moesin (ERM) complex located in the perimembranous region and involved in migration (149). There is only one evidence about iOPN in human so far. In this work the authors described that iOPN interacts with polo-like kinase-1 (Plk-1) enzyme in the nucleus, suggesting its implication in the cell duplication process (150). sOPN and iOPN participate mainly in adaptive and innate immunity, respectively. Indeed, in pathogenic conditions such as Candida infection or T-cell-mediated colitis, iOPN can promote apoptosis in myeloid progenitors through the downregulation of survivin expression and the enhancement of pan-caspase activities, while sOPN increases the size of lymphoid cells populations. Therefore, sOPN and iOPN can skew the balance between myeloid and lymphoid cells during ongoing immune responses (151).

2.2.2 Role of OPN in cancer

Although having a beneficial role in many physiological processes such as wound healing, bone homeostasis, and ECM functions, OPN has a key function in tumour-associated inflammation and metastatic dissemination. OPN is involved in acquired resistance to chemotherapy (152) and promotes cell migration and invasion, also inhibiting apoptosis of cancer cells (153). Indeed, high expression of OPN has been correlated with a more aggressive phenotype and poor survival in many cancer types (154,155).

OPN can activate the HIF-1α pathway via PI3K/Akt signalling which regulates the expression of Twist, promoting EMT in breast cancer and ovarian cancer models (156). OPN also induces the secretion of MMP2 mediated by the activation of nuclear factor kB (NF-kB), which in turn regulates melanoma cell growth, motility, and invasiveness (157). Accordingly, OPN deletion in breast cancer cells reduces the ability to grow and progress in vitro (158). In breast cancer, tumour-derived OPN can also reprogram resident tissue fibroblast toward a proinflammatory and tumour-promoting CAF phenotype (159). Furthermore, it has been demonstrated that the
transfection of OPN in murine neuroblastoma cells stimulates neovascularization, therefore linking its activity to tumour angiogenesis (160).

Besides being produced by cancer cells, OPN can also be produced by cells of the TME such as stromal or immune cells. Evidence from our laboratory suggests that whereas tumour-derived OPN protects breast cancer cells from apoptosis, myeloid cell-derived OPN, likely in its intracellular form, can shape an immunosuppressive environment at the metastatic niche (161). Other groups found that OPN in senescent fibroblast can stimulate preneoplastic cell growth and tumorigenesis in vitro and in vivo (162). Moreover, stroma-derived OPN can influence melanoma growth. Kumar and colleagues described that stromal OPN induces VEGF, ABCG2, and ERK1/2 expression by cancer cells leading to aggressive tumour growth, angiogenesis, and metastasis (163). Nemoto and colleagues demonstrated that the injection of B16 melanoma cells in OPN-deficient mice causes a reduction of metastases in bone and lung than in control mice (164). On the contrary, Bourassa and colleagues described a protective role of host OPN towards cancer, demonstrating that the lack of OPN in macrophages causes an impaired anti-tumour activity (165). Furthermore, Fan and colleagues described a protective role of iOPN towards carcinogenesis. Indeed, they described that iOPN acts as a negative regulator of the TLR pathway in macrophages reducing the release of pro-inflammatory cytokines and inhibiting liver cancer growth (147).

All these pieces of evidence highlight that OPN can exert different functions depending on tumour type/stage or different cellular sources. In many circumstances, targeting OPN could be effective for cancer therapy. On the other hand, it is fundamental to elucidate disease contexts and molecular mechanisms in which OPN operates to block tumour progression, which are not still fully defined.

In PCa OPN is associated with proliferation, metastasis, and chemotherapy resistance. Increased levels of tumour-derived OPN have been found during the progression from HGPIN to prostate adenocarcinoma and metastatic disease. Furthermore, OPN has a prognostic relevance since the increase of its expression correlates with reduced overall survival of patients (166). Castellano and colleagues observed that the levels of OPN and MMP-9 are directly correlated and increased in the plasma of PCa patients (167). Accordingly, in androgen-independent PC3 cells, upon the binding to αvβ3 integrin OPN can activate Rho GTPase signalling via RANKL leading to upregulation of CD44 and MMP-9, which in turn contribute to
ECM degradation, facilitating cell migration and metastasis (168). Furthermore, Nakamura and collaborators, demonstrated that the overexpression of OPN-b and OPN-c splicing isoforms in PC3 cells can sustain cell survival in response to docetaxel-induced cell death (169).

In apparent contrast with the above-mentioned literature, our laboratory described a protective role of OPN in limiting the emergence of NEPC in the TRAMP mouse model of PCa (170). The mechanism by which OPN can restrain NEPC onset is the object of this thesis.

2.3 MCs

MCs are potent effectors of innate immunity, originating from pluripotent progenitor cells of the bone marrow and migrating through the bloodstream in many vascularized tissues where they complete their maturation (171). The expression of CCL5 and its interaction with CCR1 and CCR4 is important for the migration of MC precursors towards tissues (172). Instead, stem cell factor (SCF)/cKit receptor axis and IL-3 have a key role in the development, maturation, and proliferation of MCs (173).

Mature MCs are mainly present in epithelial and mucosal tissues, nearby nerves, or blood vessels, within smooth muscle and mucus-producing glands, and within gastrointestinal and genitourinary tracts. However, some species, including murine rodents, present MCs also within peritoneal, pulmonary, or mesothelium cavities (171). Thanks to the presence of various receptors on their cell membrane, MCs can react to different stimuli, by releasing a plethora of chemical mediators. These can be classified as preformed mediators (histamine, proteases, tryptase, and proteoglycans), newly synthesized lipid mediators (prostaglandins, leukotrienes, platelet-activating factor), cytokines (TNFα, IL-4, IL-5, TGF-β) and chemokines (CCL2 and IL-8) (174). Preformed mediators are stored in large granules located in the cytoplasm of MCs, characterized by low pH. Every MC contains about 50-200 granules that are released a few seconds after the proper stimulus; the process is defined as MCs degranulation (175).

Murine MCs have been classified into 2 major subtypes: mucosal-type MCs and connective tissue-type MCs, according to their location. Mucosal-type MCs express chymases such as mouse MC protease (mMCP)-1 and mMCP-2, while connective
tissue-type MCs express mMCP-4, -5, -6 and carboxypeptidase A (CPA). Human MCs are instead classified based on their content of serine proteases and divided into tryptase-positive MCs (MC\textsubscript{T}), chymase-positive MCs (MC\textsubscript{C}), or both tryptase- and chymase-positive MCs (MC\textsubscript{TC}) (174). Despite these classifications, in both mouse and human tissues mixed populations of MCs have been described (176).

MCs are mainly involved in allergic diseases and inflammation. The best-studied mechanism of MC activation is the cross-linking of type E immunoglobulin (IgE) bound to their high-affinity FceRI receptors during allergic disease, which initiates a complex secretory reaction. FceRI is a heterotetrameric receptor that contains an IgE-binding α subunit, the membrane tetraspanin β subunit, and 2 disulfide-linked γ subunits that contain one immunoreceptor tyrosine-based activation motif (ITAM), important for initiating signalling pathway. When IgE is linked to the FceRI on the surface of MCs, IgE binding to the cognate antigen (Ag) causes aggregation of the receptor complex. This event induces the activation of Lyn that in turn phosphorylates ITAM and activates Syk, following ITAM binding. Lyn and Syk phosphorylate other adaptor molecules and enzymes regulating MC activation. Besides Lyn, FceRI aggregation activates a second Src family kinase, Fyn, which in turn phosphorylates Gab2 to activate the PI3K pathway (177). This mechanism triggers a complex secretory response that is characterized by the secretion of cytoplasmatic granules content such as histamine, heparin and other proteoglycans, proteases, and granule-stored cytokines. IgE/Ag stimulation also prompts the \textit{de-novo} synthesis and consequent release of lipid mediators, cytokines, growth factors, and chemokines (178,179).

MCs have also an important role in host defence against several pathogens. Indeed, MCs express several receptors such as TLRs and nucleotide-binding oligomerization domain (NOD) proteins that can recognize the pathogen-associated molecular patterns (PAMPs). Human and murine MCs can express 10 (TLR1-TLR10) or 13 (TLR1-TLR9 and TLR11-TLR13) different TLRs respectively (180). TLRs are located on the cell surface, except for TLR3, TLR7, TLR8, and TLR9 which are intracellular. Some of them form heterodimers (i.e., TLR2/TLR1, TLR2/TLR6, TLR4/TLR6) and most of them (excluding TLR3, TLR7, and TLR9) are dependent on MyD88 for their signal transduction pathway (181). One exception is TLR4, whose signalling can be either dependent or independent by MyD88. The first case
leads to the activation of NF-kB while the second one involves the adaptor proteins TRIF and TRAM initiating the type I IFN response (182).

Additionally, MCs can express a variety of receptors that, if stimulated, prompt the release of a series of pro-inflammatory cytokines and chemokines. Of importance in MCs biology is the IL-33 receptor (IL-33R), part of the TLR/IL-1R (TIR) superfamily, that when activated leads to the production of mediators including IL-1, IL-6, TNFα, CCL2, and CCL3. Furthermore, IL-33 signalling enhances the degranulation, migration, and survival of MCs (183). Other important receptors present on MCs surface are IL-18R, which leads to the production of IFNγ, GM-CSF, TNFα, and IL-1 (188), and TSLPR, which induces the secretion of IL-4, IL-5, IL-9, and IL-13 (184).

The ability of MCs to secrete different mediators according to different environmental stimuli makes them relevant players in many additional physiological functions. Moreover, their ability to influence angiogenesis, homeostasis, and inflammation and to release mediators with either pro- or anti-tumoral activity is of key relevance in cancer.

2.3.1 Role of MCs in cancer

Identified for the first time in human tumours by Paul Ehrlich, MCs have an ambivalent role in cancer, since they produce different mediators that can contribute either to promote or inhibit tumour progression (185). Several stimuli produced in the TME can activate and recruit MCs at tumour site. These include cytokines and chemokines such as IL-6, VEGF, CXCL10, CXCL1, CXCL12 and especially SCF (186).

In several tumours, MCs have a pro-tumorigenic role. In pancreatic cancer, tumour-infiltrating MCs are increased if compared to normal pancreatic tissues and correlate with a poor prognosis (187). Moreover, in a mouse model of Myc-induced beta cell pancreatic cancer, it was described that the inhibition of MC degranulation suppresses tumour angiogenesis and progression (188).

Also, in bladder cancer, the increase in the number of MCs is associated with high grade and they can enhance bladder cancer metastasis via ERβ/CCL2/CCR2 that lead to EMT and MMP9 production by cancer cells (189). The release of CXCL1/GRO-α, CXCL10/IP-10, and histamine by MCs support thyroid cancer proliferation (190).
Furthermore, MCs induce both EMT and stemness in thyroid cancer via the CXCL8/IL-8 axis, which induces Akt phosphorylation and consequent activation of Slug (191). In cholangiocarcinoma, the inhibition of histamine secretion by MCs impedes EMT and decreases cancer proliferation and angiogenesis (192). Moreover, it has been described that the depletion of MCs or the generation of chimeric mice that have defect in MCs development causes regression of preneoplastic polyps supporting the role of MCs in colon carcinogenesis (193).

By contrast, in other tumours MCs appear to have an anti-tumorigenic role. This is the case of lung cancer in which infiltration of MCs is associated with a good prognosis (194). A similar effect was observed for non-small-cell lung cancer (195), diffuse large B-cell lymphoma (196) and breast cancer (197), even if for the breast cancer the anti-tumorigenic role of MCs depends on also the carcinoma subtypes (198). In nasopharyngeal cancer it has been found that TNFα+ MCs have anti-tumour effects and correlate with a good prognosis compared to VEGFA+ MCs which have a pro-angiogenic and pro-tumorigenic roles (199). Also, in non-small cell lung cancer the infiltration MC infiltration, both MC_T and MC_TC, and their production of TNFα confers improved survival to patients (200). Indeed, an anti-tumour activity mediated by TNFα derived from MCs and its cytotoxic activity has been demonstrated exploiting the WEHI-164 TNFα sensitive cell line (201,202). In a murine model of intestinal tumorigenesis, it was discovered that MCs block adenoma formation and development, leading to apoptosis of adenoma cells (203). Oldford and co-workers unveiled that TLR2-activated MCs inhibits melanoma growth in vitro and in vivo thanks to IL-6 secretion. Furthermore, the anti-tumour action of TLR2 activated on MCs has been observed also for lung carcinoma (204).

MCs can also regulate the activation and function of other immune cells remodelling the TME and influencing either local immunosuppression or inflammation. For instance, in a murine hepatocarcinoma model, activated MCs can promote the infiltration of MDSCs through CCL2/CCR2 axis and their production of IL-17, which in turn recruits Tregs at the tumour site (205). In the transgenic APCΔ468 mouse model of colon cancer, MCs recruit MDSCs through the production of the 5-lypoxigenase, which is in turn essential to produce MC-derived leukotrienes, finally promoting intestinal polyposis (206). It is also known that activated MCs can induce the migration of effector CD8+ T-cells to the site of inflammation through the production of leukotriene B4 (207). Furthermore, the release of OPN and the
expression of co-stimulatory molecules by MCs are important for the activation and proliferation of effector CD8\(^+\) T-cells and vice versa (208). In colorectal cancer, MCs can switch the function of Tregs from anti- to pro-inflammatory, downregulating IL-10 and expressing IL-17 (209). My laboratory also demonstrated that MCs skew Tregs and effector T-cells into Th17 with a mechanism involving the secretion of IL-6 and crosstalk through the OX40L/OX40 axis (210). However, the crosstalk between MCs and Tregs is bidirectional since, via the same OX40/OX40L axis Tregs can inhibit MCs degranulation (211). Histamine released by MCs can regulate Th1 polarization of T-cells through the binding with histamine receptor type 1 but its binding to the histamine receptor type 2 can inhibit both Th1 and Th2 responses (212). Indeed, it has been found that histamine supports the immunosuppressive microenvironment through the recruitment of Tregs (213). Also, MC-derived TNF\(\alpha\) has been demonstrated to be important for T-cell activation (214). Using several cancer cell lines, Huang and colleagues have been found that SCF-activated MCs promote the immunosuppression of Tregs and NK cells through the release of adenosine (215).

In a mouse model of melanoma, it has been also observed that TLR-2-activated MCs secrete high amount of CCL3 which recruits NK cells (216). Indeed, several stimuli derived from MCs such as IL-4, IL-12, and TNF\(\alpha\) can activate NK cells (217,218). Furthermore, in addition to lipopolysaccharide (LPS) stimulation, TLR3- or TLR9-stimulated MCs promote the secretion of IFN-\(\gamma\) by NK cells (219).

The conflicting results elucidated above suggest that MCs and their mediator can have different roles, not fully unveiled yet, depending on the stage of cancer, crosstalk with other cells of TME, or their localization. Deeply studying the molecular mechanisms established between cancer cells, TME, and MCs can address these unanswered questions.
3. THE TUMOUR MICROENVIRONMENT IN NEPC

In addition to tumour intrinsic processes that guide the differentiation of adenocarcinoma towards NEPC (62), signals coming from the TME are essential in the onset and development of NEPC (220,221). For instance, it is known that hypoxia can promote EMT and NED in PCa (220). HIF-1α can cooperate with the transcription factor FOXA2 promoting the expression of several hypoxia-related target genes required for NEPC development (222). In the study of Guo and colleagues, the relationship between hypoxia and one cut homeobox 2 (ONECUT2), a driver of NEPC, was investigated. ONECUT2 activates SMAD3 that regulates the binding of HIF-1α to the chromatin, enhancing hypoxia signalling and promoting NEPC (223). Furthermore, as a second function favouring NEPC development, ONECUT2 can suppress the expression of FOXA1, necessary for AR positioning in the chromatin, therefore resulting in inhibition of the AR-dependent transcriptional in PCa cells (224).

CAFs have a role also in NEPC. Kato and colleagues described a population of CD105+ CAFs which initiate paracrine SFRP1 signalling resulting into NED of adenocarcinoma cells (225). Furthermore, epigenetic alterations can cause the silencing of RAS inhibitor RASAL3 in CAFs, starting a cascade of metabolic changes that involve glutamine synthesis, encouraging NEPC (226). In our laboratory, we also described a supportive function of fibroblasts towards NEPC. We detailed a tumour-stroma crosstalk, induced by ADT/ARPI, and activated by upregulation of GRP78 in tumour cells and we also demonstrated that the pharmacologic inhibition of GRP78 decreased the frequency of NED in castrated TRAMP mice. Mechanistically, we found that the upregulation of GRP78 triggers the release of miR-29b that in turn causes the downregulation of the matricellular protein SPARC in nearby stromal cells. Because of SPARC downregulation, fibroblasts start to secrete IL-6, leading to NED (227). Indeed, IL-6 is known to promote NEPC by triggering CgA and NSE expression via JAK/STAT3 and MAPK pathways (228) but also suppressing RE-1 silencing transcription factor (REST) (229).

It has been demonstrated that also TAM can secrete IL-6, in response to bone morphogenetic protein-6 (BMP-6) released by PCa cells, orchestrating NED in a feedback loop mechanism (230). Furthermore, Wang and colleagues showed that
TAM-derived IL-6 activates STAT3 and consequently high mobility group protein B1 (HMGB1) which interacts directly with β-catenin and enhances enzalutamide-induced NED (231). It has been described that the binding of neurotensin to its receptors NTSR1 and NTSR3 is necessary to induce NED in LNCaP cells. Furthermore, the authors described that the combination of enzalutamide and the NTSR1 antagonist, is effective in reducing NED in LNCaP xenograft-bearing mice and TRAMP mice (232).

3.1 MC roles in prostate adenocarcinoma vs NEPC

In the context of PCa, the role of MCs is also ambiguous. In vitro, they can promote the proliferation of LNCaP adenocarcinoma cells, and the occurrence of EMT via the cKit-SCF signal (233). It has also been described that infiltrating MCs can induce docetaxel and radiotherapy resistance increasing the p38/p53/p21 and ATM signals (234). Furthermore, low MCs number in needle biopsy specimens of PCa patients correlates with a good prognosis, suggesting that the number of MCs infiltrating cancer foci could be a significant prognostic factor of PCa (235). For instance, intratumoral MCs negatively regulate angiogenesis and cancer growth, whereas peritumoral MCs support the development of PCa (236,237). Also, Sullivan and colleagues described that a high number of extra-tumoral MCs is associated with disease recurrence and metastasis onset after radical prostatectomy (238). By exploiting the TRAMP mouse model (239) my laboratory unveiled that MCs could sustain prostate adenocarcinoma through two different mechanisms. Firstly, MCs can provide MMP9 to early-stage adenocarcinoma cells, necessary for cell migration and invasion [(240) and Figure 7]. Secondarily, MCs can interact with PMN-MDSCs, via CD40L-CD40 interaction, inducing upregulation of Arg1, iNOS, and STAT3 by PMN-MDSCs and thus promoting the suppression of tumour-specific CD8+ T-cells [(100) and Figure 7]. Strikingly, in the same mouse model, we discovered that the genetic or pharmacologic targeting of MCs increases the occurrence of the NEPC (240,241). This peculiar protective role of MCs against NEPC is the object of this thesis. As described in the previous chapter, my laboratory described that MCs can either promote prostate adenocarcinoma, (100,240), but inhibit the onset of NEPC, at least in the de-novo setting (240,241). Since we described a dual role of MCs in fostering the growth of adenocarcinoma but also in protecting towards NEPC, any
attempt to defeat MC in PCa should be coupled with strategies aimed at limiting NEPC outgrowth. My laboratory tried to achieve this goal by exploiting a common target expressed on both MCs and NEPC cells. Starting from the evidence that both express the c-Kit receptor, we tested a c-Kit inhibitor called imatinib, in the TRAMP mouse model. The drug restrained adenocarcinoma growth blocking MC functions, but unfortunately, was ineffective against NEPC since we discovered that these cells have a defect in c-Kit signalling (241). A second, and more successful, approach was the repurposing of the anti-epileptic drug levetiracetam, which is directed against the synaptic vesicle glycoprotein 2A (SV2A), a component of synaptic vesicles involved in exocytosis, which we found expressed by both NEPC and adenocarcinoma-infiltrating MCs. Indeed, we showed that levetiracetam can directly restrain NEPC, albeit partially, whereas the inhibition of adenocarcinoma passes through the targeting of MCs, inhibiting the secretion of MMP9. The effect of levetiracetam was confirmed both in vitro and in vivo in the TRAMP mouse model (242). Evidence collected suggest that to completely defeat PCa, another approach to abrogate pro-adenocarcinoma mechanisms and to maintain or mimic the protective effect of MCs towards NEPC is needed. This could be achieved through a better understanding of the relationship between MCs and NEPC, which is the objective of this thesis.

Fig. 7 Current knowledge of MC function in PCa. Created in BioRender.com
AIM OF THE THESIS

Patients with advanced PCa treated with ADT/ARPI usually experience relapse, and often develop NEPC as mechanism of resistance to therapy. *De-novo* NEPC can also rarely arise in a small fraction of untreated patients. Both t-NEPC and *de-novo* NEPC correlate with poor prognosis, and the lack of knowledge of molecular mechanisms that foster NEPC growth reflects the lack of suitable therapies to successfully abrogate these malignancies.

Cancer development and progression is influenced by the interaction between tumour cells with TME, whose main components are stromal and immune cells, as well as ECM proteins. Yet, the contribution of TME to NEPC onset is only marginally explored.

Among TME components, previous work of my laboratory showed that both MCs and the matricellular protein OPN seems to have a protective role against NEPC. Whereas little is known on the role of OPN in moulding prostate cancer growth, especially when of stromal origin, our data show that MCs can express OPN both *in vitro* and in tumour lesions *in vivo*. However, the role of OPN in MCs has not been fully elucidated. **Here we aim at studying TME contribution in shaping NEPC, mainly focusing on:**

- the biological role of OPN in MCs
- the molecular pathway(s) governed by MC-dervied OPN able to reduce the growth of NEPC
- the translation of the results from the murine model to the human setting
MATERIALS AND METHODS

Mice and treatments

TRAMP mice on C57BL6/J background [C57BL/6-tgN (TRAMP)8247Ng] were kindly provided by Dr. Vincenzo Bronte (Verona University Hospital, Italy), under agreement with Dr. Norman Michael Greenberg (formerly at Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Heterozygous experimental TRAMP\(^{+/\text{-}}\) mice were obtained by breeding wild-type (WT) C57BL/6 male mice and heterozygous female TRAMP mice, maintained, and screened according to ref. (239). OPN knockout mutant B6.129S6(Cg)-Spp1tm1Blh/J (OPN\(^{-/-}\)) mice were purchased from Jackson Laboratories and intercrossed over 12 generations with TRAMP mice, in order to obtain congenic B6.tgN (TRAMP)8247Ng Spp1<tm1Blh>/J (OPN\(^{-/-}\)TRAMP). Mice deficient in mast cells [C57BL/6-KitW-sh/W-sh (Kit\(^{\text{Wsh}}\); (243))] were purchased from The Jackson Laboratories and intercrossed over 12 generations with TRAMP mice, to obtain mast cell–deficient B6.tgN (TRAMP)8247Ng KitW-sh/W-sh (Kit\(^{\text{Wsh}}\)TRAMP) mice.

Every crossing give rise to 50% of TRAMP\(^{+/\text{-}}\) mice, either females or males. Every mouse was screened to determine the genotype performing PCR on a DNA sample extracted from a tale biopsy.

Experimental mice were male TRAMP\(^{+/\text{-}}\), OPN\(^{-/-}\)TRAMP\(^{+/\text{-}}\) or Kit\(^{\text{Wsh}/\text{Wsh}}\)TRAMP\(^{+/\text{-}}\) mice, sacrificed between 25 and 35 weeks of age. Because of these experimental conditions, prostate collection took at least 8 months. Furthermore, considering that the analyses of tumor phenotype required the histologic evaluation on H&E slides and assessing of NEPC markers by immunofluorescence, the time to complete the in vivo experiments reached 1 year.

Mice were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS Istituto Nazionale dei Tumori of Milan, and experiments were authorized by the Institute Ethical Committee and performed in accordance with institutional guidelines and national law (D.lgs 26/2014).

To mimic androgen deprivation therapy, surgical castration was performed on 20 weeks-old TRAMP mice, under anaesthesia with ketamine (100 mg/Kg; Imalgene, Boeringher Ingheilm) and xilazine (5 mg/Kg; Rompun, Bayer). Carprofene (5 mg/Kg; Norocarp, Norbrook) was given as analgesic after recovery post anaesthesia.
and the following day. All untreated and castrated mice were followed until 25-30 weeks of age, and then killed to collect prostates for histologic evaluation. The picture reports the timeline of all the \textit{in vivo} experiments:

![Timeline diagram]

**Bone marrow-derived mast cell differentiation \textit{in vitro}**

Bone marrow MCs were obtained by \textit{in vitro} differentiation of bone marrow cells taken from the flushing of femora and tibiae of C57/BL6, OPN\textsuperscript{-/-}, MyD88\textsuperscript{-/-} or TNFα\textsuperscript{-/-} mice. Femora and tibiae of MyD88\textsuperscript{-/-} mice were kindly provided by Prof Luigina Romani Bone marrow precursors were cultured \textit{in vitro} in RPMI 1640 (Gibco) supplemented with 20 % of foetal bovine serum (FBS; Gibco), 200 U/ml penicillin (Cambrex), 150 U/ml streptomycin, 10 mmol/L Hepes, 10 mmol/L sodium pyruvate, non-essential amino acid (NEAA) mixture, 2 mmol/L L-glutamine, 5 μmol/L β-mercaptoethanol (Gibco), in the presence of interleukin IL-3 (Peprotech, cat. no. AF-213-13) and SCF (Peprotech, cat. no AF-250-03) 20 ng/ml each, for 4 to 6 weeks. After 4 weeks of culture, MCs purity was analysing the percentage of FceRI and c-Kit double positive cells by flow cytometry (Appendix A). MCs were used for \textit{in vivo} or \textit{in vitro} experiments when purity reached at least 90%.
Mice reconstitution with Bone marrow-derived MCs.

5×10⁶ MCs were injected intraperitoneally (i.p.) into 8-week-old Kit<sup>Wsh</sup>-TRAMP mice. Mice were followed until 25-30 weeks of age, and then killed to collect prostates for histologic evaluation.

Cell lines and in vitro experiments

T23 and ST4787 cell lines were isolated from TRAMP mice as described (240,242). The MC/9 cell line (244) was kindly provided by Dr Barbara Frossi, University of Udine, Italy. All cells were cultured in DMEM (Gibco) supplemented with 10% of FBS (Gibco), 200 U/ml penicillin (Cambrex), 150 U/ml streptomycin, 10 mmol/L Hepes, 10 mmol/L sodium pyruvate, non-essential amino acid (NEAA) mixture and 2 mmol/L L-glutamine (Gibco). All cell lines were routinely tested for Mycoplasma using the MicoAlert Mycoplasma Detection Kit (Lonza, cat no. LT07-118).

For co-culture experiments, tumour cells and MCs were plated in 6-well or 12-well plates (50,000 or 10,000 cell/well respectively) at 1:1 ratio for 4 days. In dedicated experiments, 6-well transwell plates (0.4 µm pore size) were used. In this case cancer cells were seeded on the bottom of the well and MCs on the top chamber. When indicated, aTNFα neutralizing monoclonal antibody [aTNFα; 10 µg/ml, rat anti mouse, clone V1q; purified from hybridoma cells, kindly provided to our laboratory by Dr D. N. Mannel, (245)] was added in the media of co-cultures.

After 4 days of culture NEPC cells were collected, distinguished by their growth in adhesion compared to MCs that grow in suspension, for the evaluation of their growth through trypan blue cell count.

In culture experiments with tumour cells alone, when indicated, murine TNFα recombinant protein (rTNFα; 20, 50 or 100 ng/ml; Peprotech, cat n. AF-315-01A) or murine IL-1β recombinant protein (rIL-1β; 20, 50 or 100 ng/ml Peprotech, cat n.AF-211-11B) were added in the medium of cancer cells. After 4 days, cells were recovered for trypan blue cell count.
Histopathological evaluation of mouse prostates

Murine urogenital apparata were fixed in formalin and embedded in paraffin (FFPE). Sections (5μm) were de-paraffinized and re-hydrated, stained with haematoxylin and eosin (Bio-Optica). Murine prostate lesions were scored according to histopathological evaluation of a board-certified pathologist (C. Tripodo) as follows. Lesions defined as HGPIN, or initial adenocarcinoma were characterized by atypical cells forming distorted/ill-defined glands within the stroma. De-novo NEPC: sheets and nest histiology of anaplastic cells with high nuclear to cytoplasmic ratio. NED: presence of tumour foci of atypical cells with less pleiomorphic nuclei and granular chromatin, which show a tendency to diffuse growth still preserving the capability to form glandular remnants, with nuclear features of NEPC tumours (227).

Human PCa samples

FFPE human prostatectomies were taken from a cohort of PCa patients treated with neo-adjuvant ADT (bicalutamide) for more than eight months at European Institute of Oncology (IEO) in Milan, as part of a peculiar clinical protocol rendered necessary to not leave patients without treatment while surgical procedures were delayed due to the COVID-19 pandemic. Tumor samples were scored as NEPC based on expression of AR and CgA, as previously published (227). FFPE human prostatectomies of untreated PCa patients were obtained from ASST Valle Olona, Busto Arsizio. All samples were collected in accordance with the Helsinki Declaration.

Lentivirus vectors construction and viral supernatant preparation

Lentiviral vectors containing the sequences encoding either for mouse OPNf or iOPN were obtained by cloning the OPNf sequence (from pUC57-Spp1 plasmid, DBA Italia) or the iOPN sequence (kindly provided to our laboratory by Prof ML. Shinohara) into the lentiviral backbone pLVX-EF1a-IRES-ZsGreen1 (Takarabio, cat. n. 631982). The empty pLVX-EF1a-IRES-ZsGreen1 vector was used as control (scramble).
The production of lentiviral particles was performed using a third-generation packaging system involving the transfection of 293T producer cells. 5x10^6 cells were seeded in 150mm plates using 10% FBS Iscove’s IMDM (Lonza) and, the day after, a co-transfection using CaCl_2 was carried out of 4 plasmids: pMDLg/pRRE (16.25 µg), pRSV-REV (6.25 µg), pMD2-VSV-G (8.75 µg) and the plasmid with the gene of interest (OPNf or iOPN; 25 µg). After 24 hours, the supernatant was removed, and fresh medium was added. The next day supernatants containing viral particles were collected, centrifuged at 1500 rpm for 5 min to exclude residual cells and passed through a 0.45 µm filter.

**Overexpression of OPNf and iOPN in MC/9 cells**

5x10^6 MC/9 cells were resuspended in the viral supernatants and seeded in 12-well plates. After 24 h the medium was changed and, 4 days later, the percentage of infected cells was assessed by flow cytometry evaluating the percentage of GFP+ cells. After expansion, GFP+ cells were isolated by fluorescence activated cell sorting (FACS) to obtain a pure population of GFP-expressing cells. Immunofluorescence and ELISA for OPN were performed to confirm the expression of the protein.

**Flow Cytometry**

For surface staining of tumor cells or MCs, single-cell suspensions were stained with desired antibodies for 15 minutes at 4°C, then washed in PBS-2% FBS. For intracellular detection of TNFα, brefeldin A (5 µg/ml; from Penicillium brefaldianum, Sigma cat. n. B7651) was added for 4 hours and then cells were stained with surface antibodies to CD49f and CD45 to distinguish cancer cells and MC populations, respectively. Brefeldin A inhibits the trafficking from endoplasmic reticulum to the Golgi complex, thus allowing the retaining of secreted proteins into the cells and their detection by intracellular flow cytometry. Cells were then fixed with IC Fixation Buffer and permeabilized with Permeabilization Buffer according to the Intracellular Fixation & Permeabilization Set protocol (ThermoFisher Scientific, cat. n. 88-8824). Finally, the samples were stained with anti-TNFα antibody for 20 minutes at 4°C.
All samples were acquired with BD LSRII Fortessa instrument and analyzed with the FlowJo software. All the antibodies used in flow cytometry are listed in the following table:

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<th>Company</th>
<th>Clone</th>
<th>Catalogue n°</th>
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</tbody>
</table>

**Cytospin**

MCs were suspended at $10^5/100 \mu l$ in PBS. Glass slides were mounted with paper pad and cuvettes with a metal holder, loaded with 100 µl of cell suspension and then spinne[d 2 minutes at 2000 rpm with a cytocentrifuge. After detaching of cuvettes and filters, slides were dried overnight and then fixed for 15 minutes with PBS containing 4% paraformaldehyde. After permeabilization for 10 minutes with PBS containing 0,1% Triton X-100 (Sigma), sections were incubated with 0,1 M glycine (Sigma) for 5 minutes at room temperature. Then sections were blocked with PBS containing 10% of FBS and 5 µg/ml FC blocking (Anti-Mouse CD16/CD32 monoclonal antibody, Invitrogen, cat. n. 14-0161-86), we followed the protocol described for the immunofluorescence.

**Immunofluorescence**

For the detection of OPN in MCs in vitro, after the blocking, slides derived from cytospins were stained with primary antibody for 1,5 hours at room temperature and, after washing with PBS, the staining was revealed using a specific secondary antibody for 30 minutes at room temperature. Nuclei were highlighted with DAPI (ThermoFisher, cat. n. D1306) for 10 minutes at room temperature. Cover glasses were mounted using ProLong Gold antifade reagent (Invitrogen, cat. n. P36934). Fluorescent images were acquired with a confocal laser-scanning microscope Leica TCS SP8 x (Leica Microsystems). equipped with a pulsed super-continuum White
Light Laser (470–670 nm; 1 nm tuning step size). Laser lines were 495 nm for FITC and Alexa 488, 556 nm for Alexa 555. Detection ranges were 501 to 556 nm and 569 to 630 nm respectively. Images were acquired in the scan format 1024 × 1024 pixel using an HC PL APO 63X/1.40 CS2 oil immersion objective and a pinhole set to 1 Airy unit. Data were analyzed using the software Leica LASX rel.1.1 (Leica Microsystems). Images were mounted using the ImageJ 2 software.

The optimization of OPN staining protocol in MCs is described in Appendix B. For the detection of TRY, TNFα and OPN, FFPE sections (5μM) of murine and human tumour samples were de-paraffined and re-hydrated. For the double detection of TRY/TNFα and of TRY/OPN in murine samples, antigen retrieval was performed utilizing the Novocastra Epitope Retrival Solution pH9 (Novocastra, Leica Biosystem) autoclaving at 95°C for 15 minutes. For the double detection of TRY/TNFα in human samples, antigen retrieval was performed utilizing the Novocastra Epitope Retrival Solution pH6 (Novocastra, Leica Biosystem) autoclaving at 95°C for 15 minutes. Sections were brought to room temperature and washed with PBS. Then, sections were blocked with PBS containing Tween-20 (0.1%, Sigma) and 5% bovine serum albumin (BSA, Sigma). OPN and TRY primary antibodies were incubated for 1,5 hours at room temperature while the primary antibody for TNFα was incubated over night at 4°C. Secondary specific antibodies were added for 30 minutes at room temperature. Nuclei were highlighted with DAPI (ThermoFisher, cat. n. D1306) for 10 minutes at room temperature. Cover glasses were mounted using ProLong Gold antifade reagent (Invitrogen, cat. n. P36934). Images were acquired with a Leica DM4 B microscope equipped with a Leica DFC450 C digital camera, utilizing the LAS X software. Images were mounted using the ImageJ 2 software.

For the double detection of TRY and OPN in human samples antigen retrieval was performed utilizing the Novocastra Epitope Retrival Solution pH6 (Novocastra, Leica Biosystem) in a thermostatic bath at 98 °C for 30 minutes. Sections were brought to room temperature and washed with PBS. After, neutralization of Fc blocking by 0,4% casein in PBS (Novocastra, Leica Biosystem), OPN and TRY primary antibodies were incubated for 1,5 hours at room temperature. Secondary specific antibodies were added for 30 minutes at room temperature. Nuclei were highlighted with DAPI (ThermoFisher, cat. n. D1306) for the 10 minutes at room temperature. Cover glasses were mounted using ProLong Gold antifade reagent.
Images were acquired with a Zeiss Axioscope A1, and microphotographs were collected using a Zeiss Axiocam 503 Colour with Zen 2.0 software (Zeiss). Images were mounted using the ImageJ 2 software. All primary and secondary antibodies used for immunofluorescence are listed in the following tables:

### ELISA

OPN in culture supernatants was detected using the DuoSet ELISA Kit (R&D System, cat. n. DY441) according to the manufacturer protocol. Optical Densities (OD) were determined on a microplate reader Tecan Spark (Tecan).

### Multiple immunoassay

Custom ProcartaPlex Multiplex Immunoassay (ThermoFisher Scientific) plates were used to evaluate a pool of 18 murine cytokines and chemokines in supernatants derived from co-cultures between cancer cells and MCs. Samples were incubated in a 96-well plate with polystyrene magnetic beads coated with small molecule-specific antibodies and then exposed to detection antibodies. Samples were incubated with streptavidin-PE and a reading buffer was added. The plate was read in a Luminex reader (Bio-Plex-200, Bio-Rad). The concentration of each analyte bounded to its specific magnetic bead corresponds to the mean fluorescent intensity (MFI) of the reporter signal. Raw data from all the cytokines detected by the assay are included in Appendix C.
**Real time PCR**

Total RNA from cells was extracted using the Quick-RNA microprep kit (Zymo research, cat n. R1051) and its purity and yield were assessed using NanoDrop 2000c spectrophotometer (ThermoFisher Scientific). 500 ng of RNA were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, cat n. 4368814). Real time-PCR was performed in a total volume of 20 µL using the Taqman® Fast Universal PCR Master Mix no Amperase UNG (ThermoFisher Scientific, cat. n. 4352042), 20 ng of cDNA, and specific probes for Spp1 (Applied Biosystem, Mm_01611440) or Gapdh (Applied Biosystem, Mm99999915_g1) and run on a QuantStudio 3 instrument (ThermoFisher Scientific). Values were normalized to internal control (Gapdh) using the $2^{-\Delta\Delta CT}$ method.

**Analyses of mouse and human datasets**

Median normalized RNA-Seq data of the Beltran data set, including 34 CRPC samples and 15 NEPC samples (55) were downloaded from c-BioPortal (https://www.cbioportal.org/) and imported in R software (R Core Team). Class comparison between CRPC and NEPC samples (55), was performed with limma R package and consequently gene set enrichment analysis (GSEA) was performed with fGSEA R package with HALLMARK gene set, based on Normalized Enrichment Score (NES).

The MetaCore software (Clarivate Analytics, Philadelphia) was used for pathway analyses in mouse data set, generated in the lab, comparing NEPC of OPN-/- TRAMP mice [(170), GSE69903] or TRAMP mice treated with cromolyn [(240), GSE29958] and adenocarcinoma of TRAMP mice. Heatmap representations were performed using R software (R Core Team).

**Statistical analyses and reproducibility**

Statistical analyses were performed with the GraphPad Prism6 software (GraphPad Software, La Jolla, CA, USA). For *in vitro* experiments data were analysed using One-way ANOVA with Turkey’s multiple comparisons test or Wilcoxon Mann-
Whitney test. All in vitro experiments were performed at least 2 times with at least 3 biological replicates were used. Pools between independent experiments were performed.

Analysis on RNAseq data from the Beltran human data set (55) was performed using Wilcoxon Mann-Whitney test and the P-value was corrected (FDR) with the Benjamini and Hockerberg method.

For ethical reasons, the number of animals used for in vivo studies was the minimum necessary to ensure the significance of the results. The sample size is indicated in figure legends and was defined as to obtain an effect size of 0.4 with 80% power of and an error of 5% (a=0.05). We used Chi-Square Test for comparison of categorical variables indicating phenotype of tumour lesions.

In all statistical comparison, differences were considered significant when $P < 0.05$, and P-values was reported as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. 

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RESULTS

4.1 Kit<sup>Wh</sup>-TRAMP mice and OPN<sup>-/-</sup>-TRAMP mice have abnormal frequency of de-novo NEPC that can be lowered by reconstitution with MCs.

The TRAMP mouse model can mimic the pathogenesis of humans PCa. This model was produced firstly in C57BL/6 (B6) and then in FVB background and depends on the SV40 large and small T antigen (Tag) oncogene, expressed under the control of the prostate-specific rat probasin promoter (239,246,247). Probasin is regulated by androgens and when mice reach puberty, SV40-Tag oncoprotein binds and inactivates the tumour suppressor proteins TRP53 and RB1, driving tumorigenesis (239). Consequently, about 90% of TRAMP mice on a B6 background show the onset of PCa, ranging from HGPIN (between 7 and 16 weeks of age) to focal well-differentiated adenocarcinoma (≥ 16 weeks) and poorly differentiated adenocarcinoma (≥ 25 weeks). The remaining roughly 10% of TRAMP mice spontaneously develop de-novo NEPC [(100,170,227) and Figure 8)]

Fig. 8 Tumour development in TRAMP mice
Representative haematoxylin and eosin staining of prostate lesions in TRAMP mice, defined as HGPIN, Adenocarcinoma or NEPC.
Adenocarcinoma is characterized by CK8 positive atypical cells with high nuclear pleomorphism, variably margined chromatin and prominent nucleoli, which are organized in distorted glands within the stroma (227,241). Instead, NEPC lesions express NE markers like SYP and CgA and are either negative (de-novo NEPC) or positive (NEPC cases with mixed adenocarcinoma and NE features) for CK8. These lesions are arranged in sheets and nests of cells that ranges from medium-sized to large with high nuclear to cytoplasmic ratio and/or anaplastic morphology (227,242). To investigate the role of MCs and OPN in PCa, we exploited TRAMP mice on B6 background genetically deficient either for MCs, because backcrossed with MCs-deficient KitWsh mice (named KitWsh-TRAMP mice), or for OPN (named OPN−/−TRAMP mice). As already shown in previous work from my laboratory (100,170,240,241), in both strains we observed an increased frequency of de-novo NEPC, when analysed at 25-30 weeks of age (Figure 9).

**Fig. 9 Reconstitution of KitWsh-TRAMP mice with WT MCs decreases the frequency of de-novo NEPC tumors**

Percentage of prostate lesions, scored as HGPIN, adenocarcinoma (ADENO) or de-novo NEPC in 25–30-week-old TRAMP (n=15), KitWsh-TRAMP (n=15), or OPN−/− TRAMP mice (n=17). Where indicated KitWsh-TRAMP mice were reconstituted with 5x10^6 WT (n=19) or OPN−/− (n=18) MCs at the age of 8 weeks. Fisher exact test was used for the analysis of contingency between different groups. P-values are reported as: *, P < 0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001
The pharmacologic inhibition of MC degranulation with cromolyn also resulted in increased NEPC frequency (240). These data suggest that MC can control the onset of NEPC through the release of soluble factors and imply a role for OPN. To prove this hypothesis, we reconstituted i.p. 8-week-old KitWsh-TRAMP with MCs generated in vitro from bone-marrow precursors of either WT or OPN−/− mice. Histopathological evaluation of prostate lesions of mice euthanized at 25-30 weeks of age showed that the adoptive transfer of WT MCs reduced the frequency of de-novo NEPC, restoring them to the levels similarly observed in untreated age-matched control TRAMP mice. Instead, the adoptive transfer of OPN−/− MCs did not recover the incidence of NEPC observed in TRAMP mice (Figure 9). These results suggest that OPN mediates the protective role of MCs against NEPC development.

### 4.2 MCs express OPN

To prove the role of OPN in MCs, we first checked its expression by immunofluorescence in in vitro generated bone marrow-derived WT MCs, setting the staining in comparison with OPN−/− MCs used as negative control (Figure 10A, for the optimization of immunofluorescence protocol see Appendix B). From now on all the in vitro experiments will be performed with bone marrow-derived MC, if not otherwise specified.

Then we moved in vivo to assess the production of OPN by MCs infiltrating prostate in TRAMP mice (Figure 10B) and in patients (Figure 10C) with adenocarcinoma. MCs were specifically identified with tryptase (TRY) staining. We used a KitWsh-TRAMP mouse reconstituted with WT or OPN−/− MCs as positive and negative control, respectively (Figure 10B).

Our results indicate that MCs are capable to express OPN both in mouse and human PCa.
Fig. 10 MCs produce OPN in vitro and in vivo in mouse and human prostates

A. Immunofluorescence for DAPI (cyan) and OPN (red) on MCs derived from a naive (WT) and OPN−/− mice. Image acquisition with confocal microscope. The arrow indicates the positivity for OPN

B. Immunofluorescence for DAPI (blue), OPN (red) and TRY (green) on prostates of a TRAMP mouse and KitWsh−TRAMP mice reconstituted with WT MCs or OPN−/− MCs

C. Immunofluorescence for DAPI (blue), OPN (red) and TRY (green) on tumor sample collected from a patient with prostate adenocarcinoma.
4.3 WT but not OPN<sup>−/−</sup>MCS limit the growth of NEPC cells in vitro in a cell-contact fashion

Data collected so far indicate that MCs are the source of OPN that is responsible for NEPC reduction in vivo. Aimed to understand the effect of MC-derived OPN on tumor cell growth, we moved in vitro exploiting 2 TRAMP-derived tumour cell lines generated in the lab that recapitulate the different stage of PCa, and in particular:

- T23= poorly differentiated adenocarcinoma with EMT features (240)
- ST4787= NEPC (expression of NE markers) (242).

We set up several co-culture experiments with T23 or ST4787 cells in the presence of MCs, either WT or OPN<sup>−/−</sup>, evaluating cancer cell growth by trypan blue exclusion assay after 4 days. We found that regardless of OPN expression, MCs did not influence the growth of T23 cells. Conversely, WT MCs were able to significantly decrease ST4787 cell growth, whereas this effect was not measurable in the co-culture between NEPC cells and OPN<sup>−/−</sup> MCs (Figure 11).

**Fig. 11 WT but not OPN<sup>−/−</sup> MCs limit the growth of NEPC cells**

Adenocarcinoma (T23) and NEPC (ST4787) cells (50,000/well) were co-cultured in the presence of WT or OPN<sup>−/−</sup> MCs (ratio 1:1). The growth rate of cancer cells was evaluated through trypan blue cell count. Cancer cells and MCs could be distinguished thank to their grown in adhesion or suspension, respectively. Data are a pool of up to 3 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample: *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P <0.0001
As discussed in the introduction section, OPN exists in 2 different isoforms, one secreted (sOPN) and one only retained into the cytoplasm (iOPN), resulting from an alternative translation initiation site of the same transcript (OPNf) (141). As indicated, we detected iOPN in MCs by immunofluorescence (Figure 10). However, we noticed that MCs secreted a tiny amount of sOPN compared to T23 and ST4787 cells (Figure 12).

Consequently, these results prompt us to hypothesize a protective role of iOPN expressed by MCs against NEPC. To exclude the role of sOPN in blocking ST4787 progression, we repeated the described co-culture experiments between ST4787 and MCs with the 2 cell types directly in contact or separated by a transwell. The presence of the transwell abrogated the effect of MCs in reducing ST4787 growth (Figure 13). These results let us assume that the contact between ST4787 cells and MCs is necessary to restrain NEPC cell growth, excluding a relevance for secreted proteins in this phenomenon and suggesting an involvement of OPN in its intracellular isoform.

**Fig. 12 MCs produce very low amount of OPN in comparison to PCa cells**

Secretion of OPN by MCs and PCa cells measured by ELISA in culture supernatants.

One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample P-values are reported as: *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \); ****, \( P < 0.0001 \)

Consequently, these results prompt us to hypothesize a protective role of iOPN expressed by MCs against NEPC. To exclude the role of sOPN in blocking ST4787 progression, we repeated the described co-culture experiments between ST4787 and MCs with the 2 cell types directly in contact or separated by a transwell. The presence of the transwell abrogated the effect of MCs in reducing ST4787 growth (Figure 13). These results let us assume that the contact between ST4787 cells and MCs is necessary to restrain NEPC cell growth, excluding a relevance for secreted proteins in this phenomenon and suggesting an involvement of OPN in its intracellular isoform.
4.4 iOPN has a protective role towards NEPC

Aimed at verifying which isoform of OPN was relevant in blocking ST4787 cell growth, we overexpressed either OPNf (able to give production of both secreted and intracellular OPN) or iOPN in MC/9 cells, a murine MC line (244). Parental MC/9 cells are negative for the expression of OPN both at RNA and protein levels (Figure 14A, B and C).

Fig. 13 MCs need the contact with NEPC cells to affect their growth

ST4787 cells (50,000/well) were co-cultured in the presence of WT MCs (ratio 1:1) for 4 days in contact or separated by a transwell. For contact co-culture condition, cancer cells and MCs could be distinguished thank to their grown in adhesion or suspension, respectively. The growth rate of cancer cells was evaluated through trypan blue cell count. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. Data are a pool of two independent experiments P-values are reported as: *, P < 0.05; **, P <0.01; **, P <0.001; ****, P< 0.0001

Fig. 14 MC/9 cells do not express OPN both at RNA and protein level

A. Relative expression of Spp1 transcript levels (encoding for OPN) measured by real-time PCR on MCs, either WT or OPN\(^{-/-}\), MC/9 cells and PCa cells. Gapdh was used as housekeeping control.

B. Secretion of OPN by MCs either WT or OPN\(^{-/-}\), MC/9 cells and PCa cells measured by ELISA in culture supernatants. C. Immunofluorescence for DAPI (cyan) and OPN (red) in MC/9 cells. Image acquisition with confocal microscope.
To overexpress OPN, we took advantage of a lentiviral system. Firstly, we transfected packaging 293T cells with 2 different plasmids encoding for OPNf or iOPN respectively. These constructs, beside the OPNf or iOPN sequence, also expressed the fluorescent protein ZsGreen1 as a reporter bonded to internal ribosome entry site (IRES). Control 293T cells were transfected with a vector expressing only the ZsGreen1 protein (scramble). After 24h of transfection, we collected the viral supernatants from 293T cells, and we used them to infect MC/9 cells. We FACS-sorted infected cells MC/9 thanks to their expression of the ZsGreen1 fluorescent protein, to obtain stable MC/9-scramble, MC/9-OPNf, and MC/9-iOPN sub-lines. After the sorting, we checked the expression of OPN by immunofluorescence (Figure 15A) and ELISA (Figure 15B).

By immunofluorescence we verified that control MC/9-scramble cells did not express OPN, while both MC/9-OPNf and MC/9-iOPN stained positive with this technique, which detect the intracellular form (Figure 15A). Notably, MC/9-iOPN cells showed both cytoplasmatic and nuclear localization of OPN (Figure 15A). By ELISA we confirmed that MC/9-OPNf are able to secrete OPN while MC/9-scramble are not. Surprisingly, we observed that MC/9-iOPN cells were also able to release a small amount of OPN (Figure 15B). This could be due to the forced overexpression of the protein by the lentiviral system that results in a passive release in the extracellular space of an abnormal quantity of OPN. Then, we co-cultured ST4787 NEPC cells in the presence of MC/9-scramble, MC/9-OPNf or MC/9-iOPN to assess their influence on cancer cell growth. Differently from WT MCs (Figure 11), MC/9-scramble fostered the growth of ST4787 (Fig. 16).
Fig. 15 Infected MC/9 cells express OPN
A. Immunofluorescence for DAPI (cyan) and OPN (red) in MC/9-infected cell lines. Image acquisition with confocal microscope. B. Secretion of OPN by MC/9-scramble, MC/9-OPNf, MC/9-iOPN, and T23 by ELISA in culture supernatants. T23 was chosen as positive control of secretion.
Notably, MC/9-scramble do not express any amount of OPN; moreover, this is a cell line, also manipulated with lentiviral vectors. These are two important differences if compared with primary WT MCs derived from the bone marrow that we used in previous experiments, and a possible explanation for their promoting activity on NEPC cells. Nevertheless, forcing iOPN expression in MC/9 rendered them able to significantly suppress the growth of ST4787 cells (Figure 16), thus resembling the activity of WT, OPN-expressing, bone marrow-derived MCs (Figure 11). Interestingly, also MC/9-OPNf slightly reduced ST4787 growth compared to MC/9-scramble, albeit at a lesser extent if compared to MC/9-iOPN. This is not surprising as, indeed, OPNf represents the full length of the protein, and it can produce both sOPN and iOPN (as shown in Figure 15). These data prompt us to exclude a role of sOPN and enforce the hypothesis of a protective role of iOPN expressed by MCs against NEPC.

**Fig. 16** MC/9-iOPN cells resemble WT MCs in reducing NEPC cell growth

ST4787 cells (50,000/well) were co-cultured in the presence of MC/9-scramble, MC/9-OPNf or MC/9-iOPN cells (ratio 1:1) for 4 days. The growth rate of cancer cells was evaluated through trypan blue cell count. Cancer cells and MCs could be distinguished thank to their grown in adhesion or suspension, respectively. Data are a pool of 3 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. P-values are reported as: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
4.5 iOPN dictates the release of cytokines by MCs through MyD88-TLRs signalling pathway

Since iOPN is the main isoform involved in restraining ST4787 NEPC cell growth, we focused to the biological function in which iOPN is involved. As described before, iOPN acts like an adaptor protein binding to MyD88, which controls the activation of signalling pathways downstream most of TLRs, namely TLR2, TLR4, and TLR9 (141,145), regulating the secretion of several cytokines (248). Indeed, it is well known that MCs can express TLRs on their surface (180), a result that we also verified in our MCs (Figure 17). Notably, interrogating gene expression profiles (GEP) generated in the lab from our murine models (170,240), enrichment analysis showed that TLRs signalling pathways are significantly downregulated in NEPC tumours grown in either OPN–/–TRAMP mice or in TRAMP mice treated with cromolyn (that hampers MCs function), if compared with TRAMP-derived adenocarcinoma (Figure 18A-B).

Fig. 17 MCs either WT or OPN–/– express more TLR2 than TLR4
Representative histograms showing expression of TLR4 and TLR2 measured by flow cytometry in WT and OPN–/– MCs.
Fig. 18 TLRs signalling pathways are significantly downregulated in murine NEPC compared to adenocarcinoma PCa

A. Pathways significantly downregulated in NEPC tumours of OPN<sup>−/−</sup>TRAMP vs adenocarcinoma of TRAMP mice identified by MetaCore software

B. Pathways significantly downregulated in NEPC tumours of TRAMP mice treated with cromolyn vs adenocarcinoma of TRAMP mice identified by MetaCore software
To assess if TLR-mediated pathways are implicated in hampering ST4787 cell growth, we performed co-cultures between ST4787 and WT, OPN\textsuperscript{\textasciitilde}/ or MyD88\textsuperscript{\textasciitilde}/ MCs, evaluating cancer cell growth by trypan blue cell count. We discovered that, like OPN\textsuperscript{\textasciitilde}/ MCs, MyD88\textsuperscript{\textasciitilde}/ MCs did not have any effect in blocking ST4787 cell growth (Figure 19).

**Fig. 19** MyD88\textsuperscript{\textasciitilde} and OPN\textsuperscript{\textasciitilde} MCs do not affect the growth of NEPC cells

ST4787 cells (50,000/well) were co-cultured in the presence of WT, OPN\textsuperscript{\textasciitilde}/ or MyD88\textsuperscript{\textasciitilde}/ MCs (ratio 1:1) for 4 days. The growth rate of cancer cells was evaluated through trypan blue cell count. Cancer cells and MCs could be distinguished thanks to their grown in adhesion or suspension, respectively. Data are a pool of 5 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. \(P\)-values are reported as: *, \(P < 0.05\); **, \(P <0.01\); ***, \(P <0.001\); ****, \(P <0.0001\)

We hypothesized that the encountering with NEPC cells triggers in MCs the activation of TLR-mediated pathways, thus leading to the release of cytokine(s) that in turn affect tumour growth. The absence of OPN or MyD88 in MCs could alter cytokine release and therefore explain the failure of OPN\textsuperscript{\textasciitilde} and MyD88\textsuperscript{\textasciitilde} MCs in blocking NEPC cell proliferation. To investigate this hypothesis, we harvested supernatants from the co-cultures between ST4787 cells and WT, OPN\textsuperscript{\textasciitilde} or MyD88\textsuperscript{\textasciitilde} MCs to perform a multiplex immunoassay, analysing the secretion of different cytokines/chemokines, produced as consequence of TLR activation and related to MCs (Table 1 and Appendix C).

**Table. 1 Cytokines and chemokines tested on the co-culture between cancer cells and MCs**

List of cytokines and chemokines tested in the cultured media of NEPC or adenocarcinoma cells in the presence of WT, OPN\textsuperscript{\textasciitilde} or MyD88\textsuperscript{\textasciitilde} MCs through a multiplex immunoassay.
As control, we performed the same experiment with supernatants from T23 adenocarcinoma cells, alone or in presence of MCs. We found that IL-1β and TNFα were highly produced in the co-culture between ST4787 cells and WT MCs, but not when ST4787 were cultured either alone or in the presence of OPN−/− or MyD88−/− MCs (Figure 20). Notably, neither IL-1β nor TNFα were detected in cultures of T23 either alone or in the presence of any kind of MCs, suggesting that the secretion of these cytokines by MCs could be specifically induced by NEPC (Figure 20). T23 cells were indeed able to trigger different pathways in MCs, as we could detect CCL3 only in their co-cultures, regardless of whether MCs were sufficient or deficient for MyD88 or OPN (Figure 20).

**Fig. 20 IL-1β and TNFα are highly secreted by in the co-culture between WT MCs and ST4787 cells**

ST4787 or T23 cells (50,000/well) were co-cultured in the presence of WT, OPN−/− or MyD88−/− MCs (ratio 1:1) for 4 days. The culture medium was harvested, and a multiple immunoassay was performed.

### 4.6 iOPN controls the release of TNFα by MCs to restrain NEPC growth *in vitro*

Since TNFα and IL-1β were the most abundant cytokines released in the co-culture between MCs and ST4787 cells, we supposed that these cytokines can have an antiproliferative role towards NEPC. Indeed, ST4787 cells express the cognate receptors IL1R1, TNFR1 (CD120a) and TNFR2 (CD120b) on their surface (Figure 21). Therefore, we treated ST4787 cells with 3 different doses (20 ng/ml, 50 ng/ml, or 100 ng/ml) of recombinant (r) TNFα or IL-1β and after 4 days we evaluated their growth by trypan blue cell count. We observed that only rTNFα was able to restrain ST4787 cell growth, already at the dose of 50 ng/ml (Figure 22).
**Fig. 21 IL1R and TNFR (CD120a and CD120b) are expressed by ST4787**

Representative histograms showing expression of IL1R, CD120a and CD120b measured by flow cytometry in ST4787 cells.

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**Fig. 22 rTNFα restrains ST4787 cell growth in vitro**

ST4787 cells were treated with rIL-1β or rTNFα at 3 different doses (20 ng/ml, 50 ng/ml, 100 ng/ml) for 4 days. The growth rate of cancer cells was evaluated through trypan blue cell count. Data are a pool of 3 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. P-values are reported as: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001
Notably, the same amount of rTNFα was not able to reduce the growth of T23 (Figure 23A), even if they express TNFα receptors (Figure 23B), thus confirming its specific activity only towards ST4787 cells.

**Fig. 23 rTNFα is not functional towards T23 cells even if they express TNFRs**

*Fig. 23* rTNFα is not functional towards T23 cells even if they express TNFRs. **A.** T23 or ST4787 cells were treated with rTNFα (50 ng/ml) for 4 days. The growth rate of cancer cells was evaluated through trypan blue cell count. Data are a pool of 2 independent experiments. Mann-Whitney test was used for the analysis of significance between each couple of samples (T23 treated or not and ST4787 treated or not). *P*-values are reported as: *, *P* < 0.05; **, *P* <0.01; ***, *P* <0.001; ****, *P* <0.0001

**B.** Representative histograms showing expression of CD120a and CD120b measured by flow cytometry in T23 cells.

Even if results so far strongly suggest that MCs, and not tumour cells, produce TNFα, the multiplex immunoassay (Figure 20) does not allow distinguishing which of the 2 cell types was the actual source of this cytokine. To assess this issue, we relied on flow cytometry that allowed us to distinguish ST4787 and MC populations thanks to the selective expression of CD49f and CD45, respectively (Figure 24A). We confirmed that WT MCs increase the production of TNFα when cultured in presence of ST4787 cells (Figure 24B). According to previous results (Figure 20), the production of TNFα was specifically triggered by contact with NEPC cells, and only if MCs were competent for OPN or MyD88 (Figure 24B).
To further demonstrate that MC-derived TNFα is involved in blocking the growth of NEPC cells, we utilized TNFα−/− MCs for our already described (Figure 11) coculture experiment with ST4787 tumour cells. At odd with WT MCs, but similarly to OPN−/− MCs, TNFα−/− MCs were not able to hamper ST4787 cell growth (Figure 25). Furthermore, we found that adding a TNFα neutralizing antibody (aTNFα) in

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Fig. 24 WT MCs produce TNFα when they are in contact with only ST4787 cells
Adenocarcinoma (T23) and NEPC (ST4787) cells (200,000/well) were cocultured in the presence of WT, OPN−/− or MyD88−/−MCs (ratio 1:1). After 16 hours we evaluated the production of TNFα by flow cytometry.
A. Gating strategy applied to separate ST4787 (CD49f positive and CD45 negative) from MCs (CD45 positive and CD49f negative)
B. Percentage of TNFα positive MCs after co-culture. Data are representative of 2 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. P-values are reported as: *, P < 0.05; **, P <0.01; ***, P <0.001; ****, P < 0.0001
the co-culture between ST4787 cells and WT MCs the growth rate of cancer cells was restored (Figure 25).

**Fig. 25** Targeting TNFα suppressive activity of MCs against ST4787 cells

ST4787 cells (50,000/well) were co-cultured in the presence of WT, OPN<sup>−/−</sup> or TNFα<sup>−/−</sup> MCs for 4 days (ratio 1:1). Cancer cells and MCs could be distinguished thanks to their grown in adhesion or suspension, respectively. Were indicated, in the co-culture between ST4787 cells and WT MCs an aTNFα blocking antibody (V1q clone, 10 µg/ml) was added. The growth rate of cancer cells was evaluated through trypan blue cell count. Data are a pool of 4 independent experiments. One-way ANOVA with Turkey’s multiple comparisons test was used for the analysis of significance between each sample. P-values are reported as: *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P <0.0001

Then, we wanted to determine if iOPN was the main player in inducing MCs to release TNFα after the contact with NEPC cells. Thus, we performed a co-culture experiment with ST4787 in the presence of MC/9-scramble, MC/9-OPNf or MC/9-iOPN and evaluated the production of TNFα by flow cytometry. Notably, the contact with ST4787 significantly upregulated the production of TNFα in MC/9-iOPN relative to controls (Figure 26).
Altogether, these data indicate that in MCs iOPN dictates the release of TNFα via MyD88-dependent TLR signalling to counteract the growth of NEPC. These results also imply that in fully-grown, clinically detectable, NEPC, these key players should be downregulated. To prove this hypothesis, we analysed a human data set that compares patients with CRPC to patients with NEPC (55). GSEA found that the hallmark pathway “IL-6_JAK_STAT3_signaling” that includes genes like TLR2, TNF and MYD88 is significantly downregulated in NEPC tumours of these patients (Figure 27A). Furthermore, in the same data set we found that CPA3 and TPSAB1, 2 MC-specific genes, are significantly downregulated in NEPC patients (Figure 27B).

**Fig. 26 iOPN controls the release of TNFα**

**A.** Gating strategy applied to separate ST4787 from MC/9 and representative histogram showing TNFα expression in the gate of MC/9 ZsGreen1 positive cells. **B.** Quantification of staining as RFI calculated as a ratio of the MFI of the stained sample and the MFI of the fluorescence minus one (FMO) control. Data are a pool of 2 independent experiments. Mann Whitney test was used for the analysis of significance between the samples. P-values are reported as: *, P < 0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001
4.7 TNFα restrains NEPC in vivo and it is expressed by MCs in murine and human NEPC specimens

Data collected so far showed that in MCs the TLR/iOPN/MyD88 axis controls the release of TNFα to restrain the growth of NEPC cells, at least in vitro. We then further investigated the role of TNFα in vivo. According to our hypothesis that MCs prevent NEPC growth by TNFα release, our results indicate that MCs are absent (240) and Figure 27B) and TNFα is downregulated (Figure 27A) in fully-grown NEPC. Nevertheless, we also showed that TNFα production in MCs is specifically triggered by the contact with NEPC cells and not with adenocarcinoma cells (Figure 24). This let us to hypothesize that MCs are present during the initial phases of NEPC development, when they are triggered to produce TNFα. To mimic a setting
of incipient NEPC, we collected prostates from TRAMP mice subjected to surgical castration, a condition that equals ADT in mice and induces focal NE differentiation within adenocarcinoma (227). Immunofluorescence showed the presence of TNFα-expressing MCs in focal NEPC areas (Figure 28). Conversely, MCs infiltrating adenocarcinoma of untreated TRAMP mice did not show any staining for TNFα (Figure 28).
**FOCAL INCIPIENT t-NEPC AREA**

**Fig. 28 MC-infiltrating prostates of TRAMP mice with NEPC tumour express TNFα**

Immunofluorescence for DAPI (blue), TNFα (red) and TRY (green) on adenocarcinoma of untreated TRAMP mice (ADENO) or in focal t-NEPC areas of TRAMP mice subjected to surgical castration. Corresponding H&E staining is showed in the lower panels.
To validate the results obtained in our murine model, we collected prostatectomy specimens from patients that received ADT (bicalutamide) to evaluate the expression of TNFα in infiltrating MCs. These tumour samples present focal areas of NEPC, staining positive for CgA and SYP, as we previously shown (227). We observed from the immunofluorescence that in areas of incipient NEPC MCs expressed high levels of TNFα, while this did not occur in adenocarcinoma-infiltrating MCs (Figure 29).
This result corroborates our data obtained \textit{in vitro} and in the murine model, confirming that MCs in contact with NEPC cells secrete TNF$\alpha$. In turn, TNF$\alpha$ is able to counteract the growth of NEPC (Figures 22, 23, 25); therefore, we hypothesize that to fully outgrow, NEPC need to evade the TNF$\alpha$-mediated control exerted by MCs. We will explore these escape strategies in the future.

To finally assess whether MC-derived TNF$\alpha$ has a protective role in reducing the growth of NEPC \textit{in vivo}, we reconstituted 8-week-old Kit$^{Wsh}$-TRAMP mice with TNF$\alpha^{-/-}$ MCs and we sacrificed them at 25-30 weeks of age for histopathological evaluation. Notably, the frequency of NEPC in mice receiving TNF$\alpha^{-/-}$ MCs remained similar to that observed in untreated Kit$^{Wsh}$-TRAMP mice, confirming that TNF$\alpha$ is important in decreasing the growth of NEPC also \textit{in vivo} (Figure 30).

\textbf{Fig. 29 MC-infiltrating prostates of patients presenting NED express TNF$\alpha$}

\textit{Immunofluorescence for DAPI (blue), TNF$\alpha$ (green) and TRY (red) on tumor samples collected from 2 patients underwent ADT with prostate adenocarcinoma (ADENO) or t-NEPC.}
In conclusion, our data indicate that TNFα produced by MCs has a protective role in restraining NEPC. We demonstrated that iOPN dictates the release of TNFα by MCs via MyD88-dependent TLR signalling pathway activated in response to stimuli coming from NEPC, but not from adenocarcinoma cells. MC-produced TNFα leads in turn to the inhibition of NEPC growth (Figure 31).

**Fig. 30** Reconstitution of KitWsh-TRAMP mice with TNFα−/− MCs does not decreases the frequency of de-novo NEPC tumours

Percentage of prostate lesions, scored as HGPIN, adenocarcinoma (ADENO) or de-novo NEPC in 25–30-week-old KitWsh-TRAMP mice reconstituted with 5x10^6 TNFα−/− MCs (n=16) at the age of 8 weeks. TRAMP (n=15) and KitWsh-TRAMP (n=15) mice used as controls were the same showed in Figure 8. Fisher exact test was used for the analysis of contingency between different groups. P-values are reported as: *, P < 0.05; **, P <0.01; ***, P <0.001; ****, P< 0.0001

In conclusion, our data indicate that TNFα produced by MCs has a protective role in restraining NEPC. We demonstrated that iOPN dictates the release of TNFα by MCs via MyD88-dependent TLR signalling pathway activated in response to stimuli coming from NEPC, but not from adenocarcinoma cells. MC-produced TNFα leads in turn to the inhibition of NEPC growth (Figure 31).

**Fig. 31** iOPN controls the release of TNFα by MCs to restrain NEPC.

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Still open is the identification of which is the NEPC-specific stimulus that triggers the activation of TLR signalling via iOPN-MyD88 in MCs. We will investigate on this in the next future.
DISCUSSION

T-NEPC often emerges in patients as a mechanism of resistance to ADT/ARPI; however de-novo NEPC can also occur, albeit very rarely, in patients who have never undergone therapy (52,63). The mechanisms governing NEPC arise, and progress remain poorly characterized, making difficult the identification of predictive/prognostic biomarkers and the development of effective therapies. Tumour cell plasticity (55,220) has been identified as a major driver of t-NEPC, also implying a role for signals coming from the TME (220–222,225–227,230,231,240,241) in the development of these aggressive tumours. Truly, the actual contribution of TME in NEPC onset/development is still poorly understood. Due to its rarity, investigation of de-novo NEPC is more difficult. Nevertheless, having common pathways due to similar genomic alterations and transcriptomes (58), t-NEPC and de-novo NEPC could be studied even a in separate settings. Commonalities could then be exploited for therapeutic targeting of both.

In this study we unveiled a novel mechanism by which MCs counteract the growth of de-novo NEPC via iOPN-MyD88-TNFα axis. These results are in line with evidence showing that TNFα secreted by MCs has cytotoxic activity (249,250). Indeed, it has been shown that TNFα positive MCs have anti-tumour property and correlate with good prognosis in nasopharyngeal cancer (199). However, we still need to elucidate the specific activity of TNFα on NEPC cells, as it has been described that TNFα can activate either apoptosis or necroptosis pathways (251,252).

Nevertheless, our data are in apparent contrast with a study describing that, when in co-culture with PCa cells, MCs can promote their down-regulation of AR and consequently the upregulation of NSE, a NE marker (253). However, in the cited study authors used a human MC line that can have different features compared with primary MCs derived from the bone marrow. Indeed, phenotype and function of immortalized MC lines change during culturing due to accumulation of chromosomal abnormalities (254). Furthermore, the LNCaP xenograft model used by the authors does not represent a good model to evaluate the contribution of the adaptive immunity, that could also influence MCs recruitment and function. Adaptive immune cells could also have a peculiar role on NEPC. Also, we cannot exclude that MCs can exert different functions either during the adenocarcinoma-
NEPC transition fostered by ADT/ARPI or in de-novo NEPC. These issues deserve further clarifications.

Our results prompt us to rule out an involvement of sOPN, highlighting a role of MC-derived iOPN in restraining NEPC. Nevertheless, our data showing that iOPN is protective against NEPC growth may appear contradictory to literature in which OPN has a detrimental role in PCa (167,255,256). However, this harmful activity of OPN could be explained with 2 observations:

i) the above-cited studies showing that OPN promotes PCa are focused on tumour-derived OPN (167,255,256). Indeed, our data, solely related to OPN produced by the TME are in line with the study of Danzaki and colleagues that describes a protective role of NK cell derived OPN in TRAMP mice (257). However, in this study the authors excluded a role of iOPN, and they did not evaluate the rate of NEPC, focusing the attention on adenocarcinoma and metastases onset.

ii) it is important to highlight that OPN is subject to several post-translational modifications (258) and it exists as sOPN or iOPN isoform (141). These modifications or isoforms can influence the activity of OPN towards a pro-tumoral or an anti-tumoral activity.

The data provided here indicate that MC-derived TNFα is sufficient to restrain NEPC growth in vitro and in vivo and that its effect is NEPC-specific since we did not observe any TNFα activity towards adenocarcinoma cells. We also showed that TNFα is selectively produced by MCs upon encounter with NEPC cells but not with adenocarcinoma cells. However, we know from our previous studies that MCs are enriched in areas of adenocarcinoma whereas their number decrease in NEPC tumours in both TRAMP mice and human prostate cancer patients (240,241). Accordingly, in silico analyses on a human data set (55) suggest that when NEPC become full-blown all the players involved in the network that we detailed here (i.e., MCs, TNFα and TLRs related genes) are downregulated in NEPC patients compared to CRPC patients.

All these observations enforced us to think that the activity of MCs against NEPC is exerted in a precisely time frame. Indeed, both in murine and human prostate cancer samples, we found that MCs are present in areas of adenocarcinoma, but also in focal, small areas of NEPC observed either after surgical castration or after ADT, respectively. In such incipient NEPC areas, we were able to detect TNFα expressing MCs, whereas none of the MCs detected in adenocarcinoma stained
positive for TNFα. We hypothesize that MCs-derived TNFα can control NEPC outgrowth at these initial phases. Then, we speculate that tumour can adopt escape mechanisms by which MCs are inhibited of their function, excluded from the tumour area, or even eliminated. These supposed MC-escape mechanisms will be the object of future investigations.

In the next future, we will also investigate on NEPC-derived stimuli that can specifically trigger the activation of TLR signalling via iOPN-MyD88 in MCs. This could be important to discover new prognostic or predictive biomarkers for NEPC tumours.

Our results pave the way to several considerations about the possible therapeutic implications for NEPC patients. We can speculate that the adoptive transfer of MCs after ADT/ARPI treatment could represent a therapeutic opportunity to reduce the rate of t-NEPC onset. Nevertheless, while TNFα is harmful for NEPC, other function of MCs needs to be limited in the presence of residual adenocarcinoma. Understanding the pathways involved in the process of MC clearance could be of great interest in restoring MC homing or activity at tumour site. With this aim, we will exploit NEPC emerging in TRAMP mouse model which displays similar morphological and molecular properties of human NEPC (259), trusting to translate the results from the TRAMP model in novel therapeutic options for NEPC patients. TNFα administration could also represent a useful therapeutic approach for NEPC tumours. However, systemic treatment with TNFα causes high toxicity (260–262) thus, it is not suitable for a clinic setting so far. The development of targeted delivery systems of TNFα could be a novel frontier for localized therapy in NEPC patients, reducing systemic toxicity and implementing the efficacy.

Also, TLR agonists could be used as adjuvant agents for immunotherapy in patients undergoing ADT/ARPI. It is known that iOPN acts downstream TLR2, TLR4 and TLR9. Our *in vitro* data showing that MCs need to contact NEPC cells to produce TNFα likely excluded the implication of TLR9, expressed only at the intracellular level. Moreover, having found that MCs have more TLR2 than TLR4 on cell surface, we hypothesize an involvement of TLR2 in the iOPN-MyD88-TNFα axis here unveiled. Notably, Oldford and colleagues demonstrated that the TLR2 agonist Pam3CSK4 stimulate MC-derived TLR2 inhibiting activity against tumour growth *in vivo* (204).
An agonist for TLR2 and TLR4 was approved by the Food and Drug Administration for intravesical bladder cancer (263). Other TLR2 agonists such as CBLB612 or ISA-20 were tested in phase 2 clinical trials for cancer therapy (NCT02778763 or NCT02821494 respectively) but the results of these trials have not been published yet and data about toxicity or side effects are not available. Furthermore, several cancer cells express TLRs (264), making the role of certain TLRs in cancer therapy controversial.

In conclusion, we demonstrated that iOPN dictates the release of TNFα by MCs via TLR-MyD88 signalling pathway activated in response to stimuli specifically coming from NEPC cells. In turn, MC-derived TNFα leads to the inhibition of NEPC growth. Our data pave the way to further investigations about the protective role of MCs in de-novo and t-NEPC, foreseeing the possibility to identify novel biomarkers and therapeutic targets for these lethal forms of prostate cancer.
APPENDIX

A. Evaluation of purity and maturation of MCs.

As previously described in the material and method section, after 4 weeks of culture, MCs purity was analysed evaluating the percentage of CD11b negative and FcεRI and c-Kit double positive cells by flow cytometry. Here, is a representative analysis.

B. MC-derived OPN immunofluorescence staining protocol optimization.

OPN immunofluorescence staining protocol in MCs required several attempts. Firstly, we performed an experiment to choose the specific antibody for immunofluorescence technique:

- Goat anti-Osteopontin antibody (Sigma Aldrich, cat.n. O7635)
- Rabbit anti-Osteopontin antibody (Abcam, cat. n. ab8448)
- Rabbit anti-Osteopontin antibody (Abcam, cat. n. ab218237, produced recombinantly, animal-free).

We fixed the cells using 4% paraformaldehyde solution (Sigma) for 10 minutes and permeabilizing them with 0,1% Tryton-100 (Sigma) in PBS for 10 minutes. Then, we tried to avoid non-specific antibody binding using 0,1M glycine.
(Sigma) in PBS for 5 minutes and 2% BSA in PBS for 1 hour as blocking solution. We finally avoided non-specific background using 10% FBS and CD16/32 (ThermoFisher Scientific, cat. n. 14-0161-86) for 30 minutes as blocking solution and we continued the staining with primary antibody, secondary antibody and DAPI as described in the material and method section.

C. **Multiple cytokine assay**

Here we report the raw data of all other cytokines detected by the multiple immunoassay, not included in figure 20. Besides TNFα and IL-1β (Fig. 20), we also detected increase of IL-4 in the coculture between ST4787 and WT MCs. However, differently from rTNFa (Figure 22), mouse recombinant IL-4 (rIL-4; Peprotech, cat. no. AF-214-14) was not able to reduce the growth of ST4787 cells (red square) and was further excluded from our hypothesis.
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I would like to thank several people that have supported me in these great 4 years of my PhD path. Each of them has contributed to reaching this important goal leading to my personal and professional growth.

First, I want to thank Dr. Elena Jachetti. She believed in me immediately, contributing to the development of my critical thinking and my professional growth as a scientist. I will never forget your reprimands (some were very scary) that pushed me to do more, feeling free to express my ideas. You’re my mentor and I hope our paths will never diverge.

Thanks to Dr Mario Paolo Colombo who supports me every day of my lab life. Thanks for all our scientific discussion and for giving me the opportunity to increase my scientific background encouraging me to participate in several international meetings and apply for prizes and fellowships. I’m very happy to develop my scientific career in your lab.

A special thanks goes to Prof Carlo Pucillo and Dr Barbara Frossi because their contribution was precious to me. Thank you for your advice and encouragement. I hope that our collaboration can last for a long time.

Many thanks to Prof Claudio Tripodo, Dr Valeria Cancila, Dr Giuseppe Renne and Dr Marco Bregni for the pathological evaluations and for their valuable help in the procuring of human samples.

Thanks to the coordinator of The Open University PhD committee Dr Luca Roz for the enthusiasm and care dedicated to this PhD programme and its students.

Thanks to Renata Ferri, my lab mommy, who is always loving and caring about me, and all to my colleagues and friends Claudia, Irene (Irina), Bianca, Celeste, Patrizia, Silvia, Barbara, Caterina, Irene, Annamaria, and Andrea for the thousand laughs and the love that you gave me during these years. I will never forget it.
A special thanks to Elisabetta, Viviana, Chiara, Valeria, Michela and Franci. Without your important advice, I would not be at the end of this PhD. I will always be grateful to you.

All my gratitude goes to my parents because without their continuous support I couldn’t have realized all my projects. Thanks to my sister, aunts, uncles, and cousins who believe daily in me. Thank you, grandma, for always praising me, even when I was emotionally down, and for giving me the right charge to move on. Love you all.

A proper thank to Valerio, one of the most important people in my life. You’re my strength, my joy, and the relief in my dark days. Thank you for all your encouragement, your hugs, and your patience. Love you so much.

Thanks to my Milanese home, made of beautiful people who really care about me. Sara, Giam, Andrea, and Gimmi thanks for living still with me, I know that sometimes it’s difficult! Thanks for relieving me in heavy moments with jokes and smiles. I Love you.

Thanks to my beautiful girls Betta, Marti, Flo, Merry e Cate because you have been by my side for at least 20 years and we’re always here, sharing the best and the worst, unite by this unshakeable bond which I hope lasts forever. Love you girls, you are my safe place

Thanks to all my friends Nunni, Dani, Ale, Georgis, Gabri, Marci, Enzo, Lalli, Chiari, Fra, Adi, Cami, Fra Schio, Sofia, Carmen, Giovanni, Nina, Linda, Giada, Ale, Piddu, Andre, and Colo. Thank you for the beautiful moments passed together! I’m very lucky to have people like you in my life!

Last, I want to thank my teacher Simon and my theatre companions of "Campo Teatrale", because you were my wonderful discovery of this incredible last year. Berry, Bibi, Davide, Dario, Matteo, Auri, Dani, Guillaume, and Yuri, you’re the beautiful souls in which I have loved to immerse in the deep blue. I hope that the second year is great and magical. Thank you, Simon, for having revealed to me the
hidden part of myself and thank you Vale (always vice-teatro) to have rendered more exciting our journey.

Thanks to the two little stars that shine bright every night for me. You’re in my heart.