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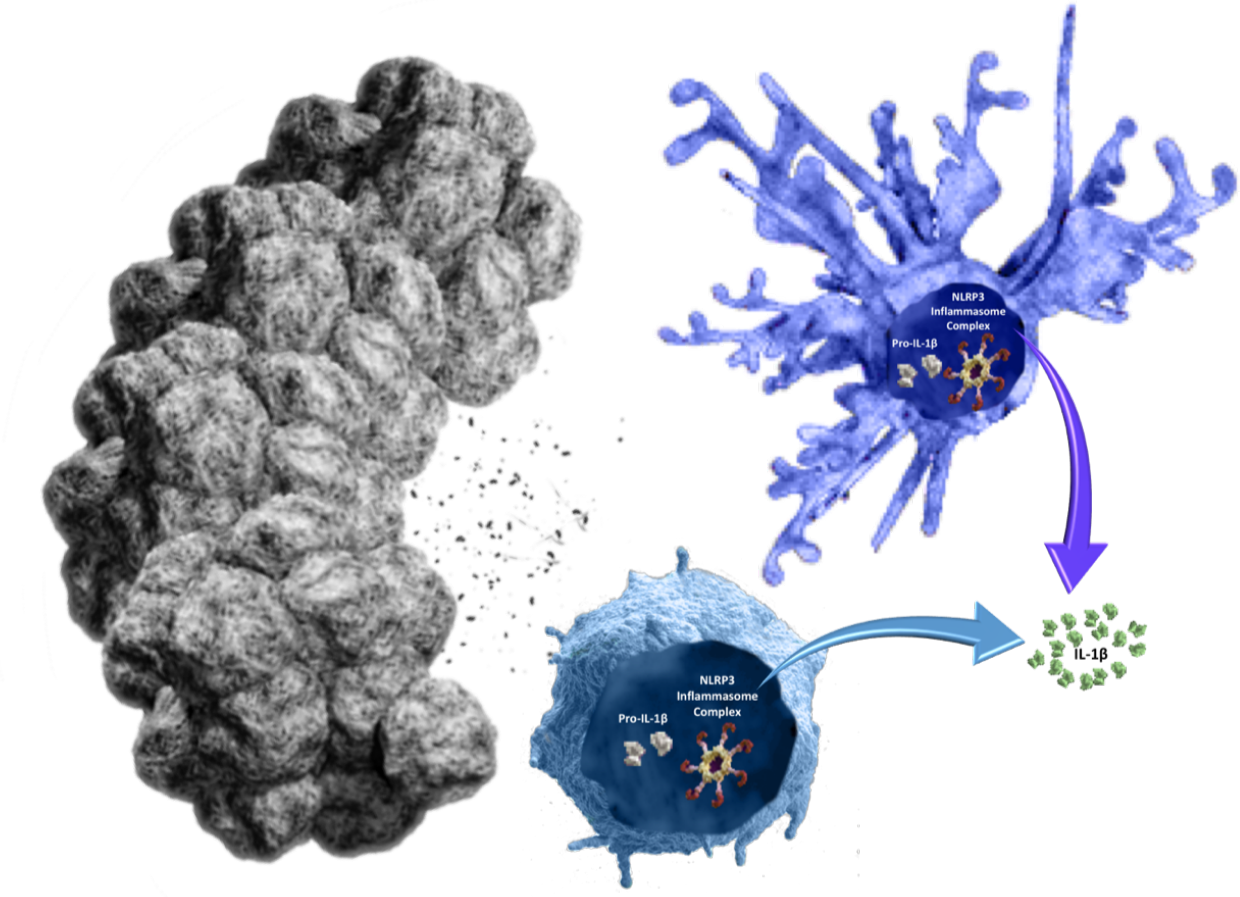
Master Thesis:

The role of inflammasome during immunotherapy responses in cancer



A Thesis for the Degree of International Master of Science (M.Sc)
Program of the University of Crete Medical School:
"Molecular Base of Human Disease"

Author:
Iosif Papafragkos, B.Sc. (AM: 1060166)

Thesis Supervisor:
Dr. Panayotis Verginis

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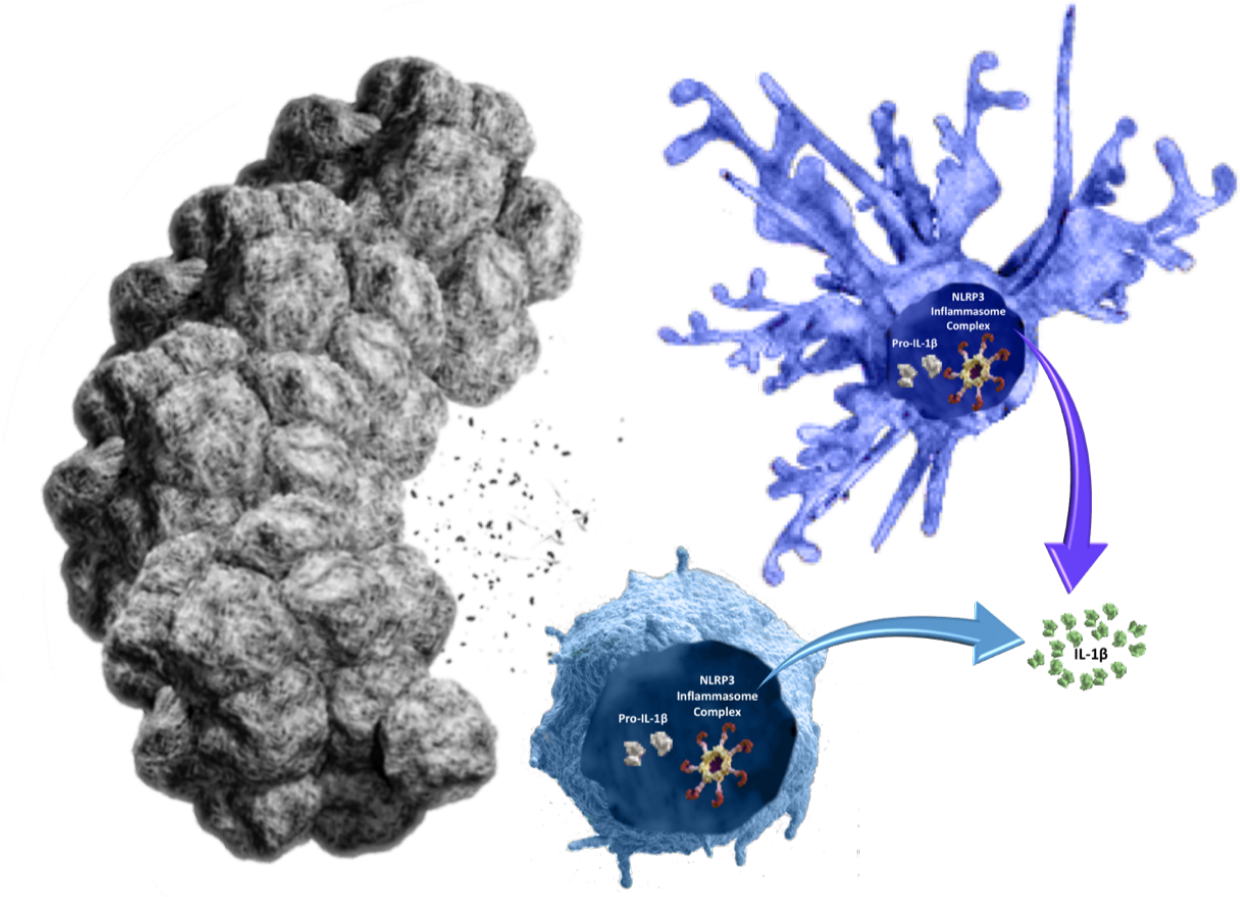
Πανεπιστήμιο Κρήτης
ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ ΣΧΟΛΗΣ

Μεταπτυχιακή εργασία:

Ο ρόλος του φλεγμονοσώματος κατά τη διάρκεια των ανοσοθεραπευτικών αποκρίσεων στον καρκίνο



Μια Εργασία για το Μεταπτυχιακό Δίπλωμα Ειδίκευσης (M.Sc)
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Συντάκτης:
Ιωσήφ Παπαφράγκος, B.Sc. (ΑΜ: 1060166)

Επιβλέπων:
Dr. Παναγιώτης Βεργίνης

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Supervisor: Panayotis Verginis, PhD, Researcher C' (BRFAA)

Three-member committee of inquiry:

- **P. Verginis**, Researcher C', Biomedical Research Foundation of the Academy of Athens (BRFAA)
- **D. Kardassis**, Professor of Biochemistry, Department of Basic Sciences, University of Crete Medical School
- **G. Bertsias**, Assistant Professor, Department of Internal Medicine, University of Crete Medical School

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Iosif Papafragkos

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Abstract

Chronic inflammation is considered one of the hallmarks of cancer initiation and progression and is a critical modulator of carcinogenesis through secretion of inflammatory cytokines, which leads to the formation of an inflammatory and immunosuppressive tumor microenvironment (TME). Chronic inflammation in TME in tumor-bearing patients has been accused to induce the accumulation and retention of highly immunosuppressive myeloid cells, such as myeloid-derived suppressor cells (MDSCs), through the aberrant activation of myelopoiesis resulting in their expansion and recruitment. In consistence with this, the highly inflammatory and immunosuppressive TME becomes a hotbed of immunologic activity promoting tumor growth and development, as it provides an immunosuppressive shield that protects the tumor from patient's immune system and immunotherapy. In this process, the inflammasome plays a crucial role. The inflammasome constitutes an innate immune sensor that regulates and controls the homeostatic innate immune pathways and is a critical for the production of active IL-1β, a potent inflammatory cytokine. Although inflammasomes are essential for host defense against pathogens and contribute to autoimmune diseases, their role in tumor progression remains controversial. Inflammasomes and their effectors are able to shape the tumor milieu through their contribution to inflammation and immune responses, affecting thus the development, progression and treatment of cancer, which depicts the diverse roles of inflammasomes in modulating carcinogenesis and their potential targeting in translational research.

For the present Master Thesis, we aimed to investigate how the NLRP3 inflammasome and consequently IL-1β shapes tumor growth, anti-tumor immune response and immunotherapy responses. Herein, we provide evidence for the crucial role of the NLRP3 inflammasome pathway in the establishment of melanoma. We demonstrate a pro-tumorigenic role for inflammasome's effector IL-1β cytokine, whose presence in TME and peripheral lymphoid organs of melanoma-bearing mice is associated with tumor progression and development. Activation of the NLRP3 inflammasome pathway in myeloid cell compartment provides an inflammatory microenvironment promoting melanoma tumor progression. Finally, our data suggest that inhibition of NLRP3 inflammasome attenuates melanoma tumor growth and alters the immune cell milieu. Collectively, these findings delineate a pivotal role of NRLP3 inflammasome in carcinogenesis and offer a better mechanistic insight of how it influences tumor progression and anti-tumor immune responses, which will undoubtedly open new avenues for translational research and the development of more efficacious immunotherapeutic approaches.

Περίληψη

Η χρόνια φλεγμονή θεωρείται ένα από τα χαρακτηριστικά γνωρίσματα της έναρξης και της εξέλιξης του καρκίνου και είναι ένας κρίσιμος ρυθμιστής της καρκινογένεσης μέσω της έκκρισης φλεγμονωδών κυτοκινών, φαινόμενο το οποίο οδηγεί στο σχηματισμό ενός φλεγμονώδους και ανοσοκατασταλτικού μικροπεριβάλλοντος του όγκου. Σε ασθενείς με όγκο η χρόνια φλεγμονή στο μικροπεριβάλλον του όγκου έχει κατηγορηθεί ότι προκαλεί τη συσσώρευση και διατήρηση σε αυτό εξαιρετικά ανοσοκατασταλτικών μυελικών κυττάρων, όπως τα κατασταλτικά κύτταρα της μυελικής σειράς (MDSCs), μέσω της μη φυσιολογικής ενεργοποίησης της μυελοποίησης που έχει ως αποτέλεσμα την επέκταση και την στρατολόγησή τους. Σε συνάρτηση με αυτό, το εξαιρετικά φλεγμονώδες και ανοσοκατασταλτικό μικροπεριβάλλον του όγκου καθίσταται πυρήνας ανοσολογικής δραστηριότητας που προάγει την ανάπτυξη και εξέλιξη όγκου, καθώς παρέχει μια ανοσοκατασταλτική ασπίδα που προστατεύει τον όγκο από το ανοσοποιητικό σύστημα του ασθενούς και την ανοσοθεραπεία. Σε αυτήν τη διαδικασία, το φλεγμονόσωμα παίζει έναν κρίσιμο ρόλο. Το φλεγμονόσωμα αποτελεί έναν αισθητήρα που ρυθμίζει και ελέγχει τα ομοιοστατικά μονοπάτια της έμφυτης ανοσίας και είναι σημαντικό για την παραγωγή ενεργούς IL-1β, μιας ισχυρής φλεγμονώδους κυτοκίνης. Παρόλο που τα φλεγμονοσώματα είναι απαραίτητα για την άμυνα του ξενιστή έναντι παθογόνων και συμβάλλουν σε αυτοάνοσες ασθένειες, ο ρόλος τους στην εξέλιξη του όγκου παραμένει αμφιλεγόμενος. Τα φλεγμονοσώματα και οι τελεστές τους είναι σε θέση να διαμορφώσουν το περιβάλλον του όγκου μέσω της συμβολής τους στη φλεγμονή και στις ανοσολογικές αποκρίσεις, επηρεάζοντας έτσι την ανάπτυξη, την εξέλιξη και τη θεραπεία του καρκίνου, γεγονός το οποίο απεικονίζει τους διαφορετικούς ρόλους των φλεγμονοσωμάτων στη ρύθμιση της καρκινογένεσης και την πιθανή στόχευσή τους στη μεταφραστική έρευνα.

Για την παρούσα Μεταπτυχιακή Εργασία, στόχος μας ήταν να διερευνήσουμε πώς το NLRP3 φλεγμονόσωμα και κατά συνέπεια η IL-1β διαμορφώνει την ανάπτυξη του όγκου, την ανοσοαπόκριση ενάντια στον καρκίνο και την ανοσοθεραπεία. Σε αυτήν την μελέτη, παρέχουμε στοιχεία για τον κρίσιμο ρόλο του μονοπατιού του NLRP3 φλεγμονοσώματος στη εγκαθίδρυση του μελανώματος. Δείχνουμε έναν ογκογόνο ρόλο για την IL-1β κυτοκίνη του φλεγμονοσώματος, της οποίας η παρουσία στο μικροπεριβάλλον του όγκου και στα περιφερειακά λεμφικά όργανα ποντικών που φέρουν μελάνωμα σχετίζεται με την εξέλιξη και την ανάπτυξη του όγκου. Η ενεργοποίηση του μονοπατιού του NLRP3 φλεγμονοσώματος στα μυελικά κύτταρα παρέχει ένα φλεγμονώδες μικροπεριβάλλον που προάγει την εξέλιξη του μελανώματος. Τέλος, τα δεδομένα μας δείχνουν ότι η αναστολή του NLRP3 φλεγμονοσώματος εξασθενεί την ανάπτυξη του μελανώματος και μεταβάλλει το προφίλ των κυττάρων του ανοσοποιητικού. Συλλογικά, αυτά τα ευρήματα περιγράφουν έναν κεντρικό ρόλο του NLRP3 φλεγμονοσώματος στην καρκινογένεση και προσφέρουν μια καλύτερη μηχανιστική εικόνα για το πώς επηρεάζει την εξέλιξη του όγκου και τις ανοσοαποκρίσεις ενάντια στον καρκίνο, ανοίγοντας έτσι αναμφίβολα νέους δρόμους για την μεταφραστική έρευνα και την ανάπτυξη πιο αποτελεσματικών ανοσοθεραπευτικών προσεγγίσεων.

1. Introduction

Inflammation, as has been reported from several years ago, is a host defense system that is aimed to guard the whole organism from both infections (protozoal, fungal, bacterial and viral) and internal attacks[1, 2]. This protective mechanism ought to be strictly regulated and controlled as in each case affects the whole homeostasis of the host's immune system and its possible deregulation leads to pathological conditions. Thus, an acute inflammation results in the elimination of the pathogenic events and the restoration of both damaged cells and tissues via the recruitment of immune cells and the secretions of inflammation's mediators, while a chronic inflammation contributes to a sequence of pathological conditions, such as autoinflammatory and autoimmune diseases, diabetes, arthritis and cancer.

The involvement of inflammation in cancer development and promotion is well-known. An inflammatory milieu is regarded to be the hallmark for cancer establishment and progression, as epidemiological studies suggest that chronic inflammation predisposes to the development of human cancers by 20%[3]. Many studies reveal that inflammatory mediators are one of the main constituents of tumor microenvironments having many tumor-promoting effects, from the resistance to growth inhibition and angiogenesis to metastasis and the escape from apoptotic cell death[3, 4]. In more detail, these tumor-promoting effects are the results of the heterogenous tumor-inducing microenvironment, which is composed from cancer cells themselves, infiltrated inflammatory cells with immunosuppressive nature and soluble factors like cytokines and chemokines.

As perceived, the inflammatory profile at the tumor site, regarding the leukocyte population size and phenotype and the behavior of the cancer cells themselves, is of great importance to understand tumor development. Of note, despite their dual role on anti-tumor immunity and their vague pathophysiological role, in cancer patients a significant increase in the levels of IL-1β[5, 6, 7] and IL-18 pro-inflammatory cytokines have been detected and their immunosuppressive nature have also been reported[8, 9]. Thus, all these stimuli and endogenous substances, which are released from the damaged and dying cells, cancer cells and non-cancer cells and compose the tumor microenvironment, have the ability to promote tumor development via the induction of chronic inflammation that results in the loss of homeostasis and facilitates tumor progression. All these "immune attacks" are sensed by a central mechanism that drives inflammation in immune cells, called inflammasome.

1.1 Tumor Microenvironment

Cancer according to the World Health Organization (WHO) is one of the most frequently occurring diseases in the world, and it is estimated that in 2018, about 9.6 million cancer-related deaths occurred[10]. However, despite years of research, deciphering the precise mechanisms by which cancer manifests is still a challenge for translational research. Carcinogenesis is a process orchestrated by a wide range of genetic and epigenetic alterations, which confer unique characteristics and properties to cancer cells, including hyperproliferation, replicative immortality, resistance to cells death, invasiveness, evasion of the immunosurveillance, and metastatic potential[11]. Nevertheless, the regulation of cancer development is not only dependent on the properties of cancer cells alone, but also needs an abetting microenvironment to contribute to the most of the features of these cells and favor malignant progression. Thus, the understanding that tumor is not only a collection of relatively homogenous cancer cells but it is recognized as organ whose biology is not limited in the elucidation of cell-autonomous properties of these cells, resulted in notion that the deciphering of its establishment mechanism requires the studying of individual specialized cell types within this abetting microenvironment that they construct during tumorigenesis and is called "tumor microenvironment".

The tumor microenvironment (TME) has first been noted by Paget *et al.*[12] over 100 years ago proposing the theory of "seed and soil", in which every single cancer cell should be regarded as a seed, alive and capable of development, but it can only live and grow if it falls on congenial soil, the tumor microenvironment. The TME (**Figure 1**) is composed of the cellular multitude which includes non-cancer cells, such as fibroblasts, endothelial cells, adipocytes, pericytes and immune cells from the lymphoid and myeloid lineage[13, 14, 15], and the soluble compartment including growth factors, chemokines, cytokines, matrix metalloproteinases (MMPs) and ROS[16, 17, 18]. Once the tumor established in host, has the ability to subvert the host's immune system and take advantage through the conversion of cancer-killing cells of TME to tumor-protector cells, hidden thus from tumor immunosurveillance and, by extension, from immunotherapy. The TME is characterized by dominant immunosuppression through the secretion of chemokines and cytokines from tumor-protector cells, preventing thus the effector cells to enter the tumor. Immunophenotyping of the immune infiltrated cells in the TME of patients, revealed significant differences in the composition of TME within patients of the same cancer types,

especially in immune cells from the myeloid lineage[19, 20, 21, 22, 23]. The myeloid cells infiltrated into tumors are very heterogenous in nature and consisted of mononuclear cell populations, including myeloid-derived suppressor cells (MDSCs)[24, 25, 26, 27], tumor-associated macrophages (TAMs)[28, 29, 30] and dendritic cells (DCs)[31, 32], and polymorphonuclear granulocyte cell populations, including tumor-associated neutrophils (TANs)[33, 34].
Additionally, the inflammatory microenvironment is an essential component for tumors[35, 36, 37, 38]. In normal tissues, myeloid cells are the gatekeepers of the immune system and provide a first line of defense by inhibiting or eliminating dangers such as pathogenic and viral infections with an acute inflammatory response, via a central signaling axis of the immune system, which is primarily expressed by myeloid cells, called inflammasome. In TME, the endogenous stimuli released by dying cells, cancer and non-cancer cells promote an excessive and uncontrolled inflammation that damages the host immunity, which in turn fails to resolve itself, resulting in a chronic inflammatory state that has a huge contribution to tumorigenesis, with the underlying molecular mechanism still remaining unclear. Chronic inflammation in TME in tumor-bearing patients has been accused to induce the accumulation and retention of highly immunosuppressive myeloid cells, such as MDSCs[39]. In consistence with this, the highly inflammatory and immunosuppressive TME promote tumor growth and development through the aberrant activation of myelopoiesis resulting in the expansion and recruitment of immature myeloid cells[39, 40], a process that has been recently reported to be also regulated by the inflammasome, which has been proposed to be required for myeloid differentiation[41]. Moreover, the immunologic profile of chronic inflammation is characterized by inflammatory and immunosuppressive cytokines, chemokines, growth factors, MMPs, reactive oxygen and nitrogen species that help to subvert that patient's immune system[17]. For example, pro-inflammatory cytokines such as IL-1, which includes two subtypes of IL-1α and IL-1β, and IL-6 have been reported to promote cancer cell invasion[42], whereas the IL-17 in TME has been linked with the limiting of anticancer immunity[43] and tumor necrosis factor-α (TNF-α) with the proliferation and differentiation of myeloid precursors into MDSCs and thus the immunosuppression[44]. Moreover, chemokines in tumor niche such as monocyte chemotactic protein-1, macrophage inflammatory protein-1α,β, and IL-8 are dedicated to promote the tumor progression and development[45, 46]. Growth factors in TME like VEGF, PDGF, EGF, TGF-β and FGF have been suggested to be mediators of cancer cell proliferation and survival[47]. Molecule as MMPs, which contribute in tumor cell invasion by extracellular matrix degradation[48], and reactive oxygen and nitrogen species[49], whose accumulation results in DNA damage, are also act as supporters to malignancy at different levels. So, it is clear that all these inflammation-associated cells and molecules contribute to the process of cancer cells proliferation, invasion, and metastasis directly.

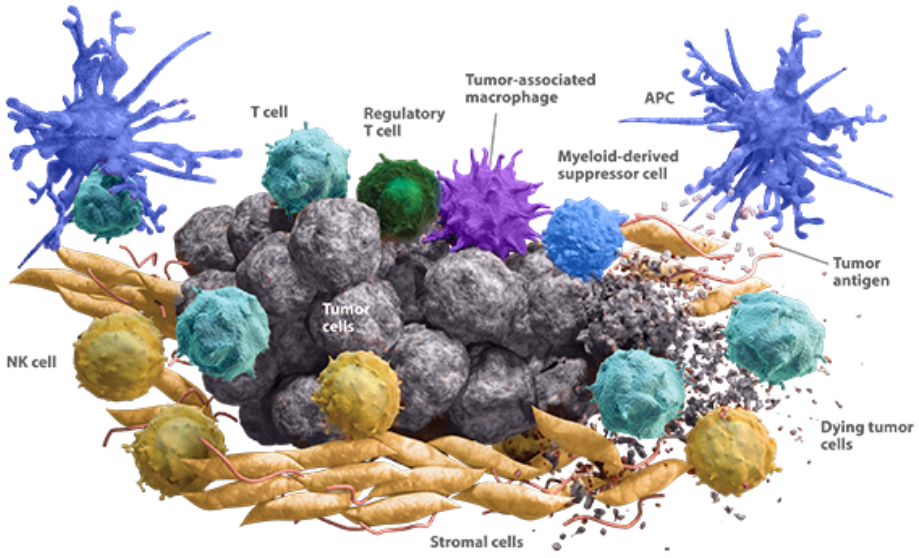

Figure 1: *Tumors have an "organ-like structure" and are composed of a cellular multitude which surround the cancer cells, and all these constitute the Tumor Microenvironment.*

1.1.1 Myeloid Derived Suppressor Cells

Among the major cell constituents of the TME are the Myeloid Derived Suppressor Cells (MDSCs), which have been characterized as the 'queen bee' of TME. MDSCs are a heterogeneous cell population and as its name reveals has a myeloid origin but is distinct from terminally differentiated mature myeloid cells, with its main functional trait be the immunosuppressive activity and its activation program (pathologic activation) be different from that of mature myeloid cells. Abnormal differentiation and function of myeloid cells is a hallmark of cancer. Thus, MDSCs have been demonstrated to be implicated not only in cancer pathogenesis, growth, and metastasis[39], but also in various aspects of immune regulation, including chronic inflammation, infectious diseases, autoimmune diseases, trauma, graft versus host disease and so on[50, 51, 52, 53]. Under steady-state physiological conditions, no or very few MDSCs are observed, whereas in cancer and other pathological processes their generation, which happens in the bone marrow, is depended from the overproduction of three growth factor molecules that participate in biological defense mechanisms, the granulocyte-macrophage colony-stimulating factor (GM-CSF) that under normal conditions drives myelopoiesis, and granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF), which under physiological conditions induce the differentiation of granulocytes and macrophages, respectively[54]. The accumulation of MDSCs is a complex process and is governed by two groups of interconnected signals. The first group of signals contributes to the expansion of immature myeloid cells and is controlled by factors that are produced by tumors or bone marrow stroma due to the inflammatory nature of tumors, including growth factors, such as GM-CSF, G-CSF, M-CSF, Stem cell factor (S-SCF), VEGF[55, 56]; transcription factors, like STAT3, STAT5, IRF8, C/EBP-β and NOTCH[57]; and other factors, such as adenosine receptor A2b, cytoplasmic receptor NLRP3, retinoblastoma protein 1 (RB1), and alarmins S100A9 and S100A8[57]. The second group of signals is needed for MDSCs' pathologic activation and is mediated by inflammatory cytokines and damage-associated molecular patterns (DAMPs) that are components of TME, including interferon-γ (IFN-γ), IL-1β, IL-4, IL-6, IL-13, TNF, and the TLR ligand HMGB1[57, 58].

The phenotypic and morphologic heterogeneity of MDSCs made it difficult to distinguish and characterize these cells. However, a recently published review came to address this issue and suggest characterization standards in burgeoning field of MDSC research[59]. Thus, it is now commonly accepted that MDSCs, in mice, are broadly identified as the cells coexpressing the Gr-1 and CD11b markers ($CD11b^+Gr1^+$). Although useful in identifying MDSC, two major subsets have been shown to exist based on their phenotypic and morphological features: monocytic MDSCs (M-MDSCs), which are morphologically and phenotypically similar to monocytes and are also characterized as $Gr\text{-}1^{lo}$ cells, and polymorphonuclear (PMN-MDSCs), which are morphologically and phenotypically similar to neutrophils and are also characterized as $Gr\text{-}1^{hi}$ cells depending on the variable expression of Gr-1 marker[55]. However, Gr-1 marker is not a singular molecule, but consists of the Ly6C and Ly6G markers and thus the MDSC subsets can be characterized based on the expression of Ly6C and Ly6G markers: PMN-MDSCs as $CD11b^+Ly6C^{lo}Ly6G^+$ and M-MDSCs as $CD11b^+Ly6C^{hi}Ly6G^-$[60, 61, 62] (**Table 1**). In humans, MDSCs are identified in the low-density Ficoll-gradient fraction of peripheral blood mononuclear cells peripheral blood mononuclear cell (PBMC). As PMN-MDSCs are defined the $CD11b^+CD14^-CD15^+$ or $CD11b^+CD14^-CD66b^+$ cells, whereas the M-MDSC can be separated according to the expression of MHC class II molecules and are defined as $CD11b^+CD14^+HLA\text{-}DR^{-/lo}CD15^-$ cells[59] (**Table 1**). CD33 myeloid marker can be also used as an identification marker for MDSCs instead of CD11b due to the fact that very few $CD15^+$ cells are $CD11b^-$ and thus M-MDSC are $CD33^+$, while PMN-MDSC are $CD33^{dim}$ [63]. There is also a population that is called "early-stage MDSCs" (eMDSCs), which has been defined as immature MDSCs containing mixed group of MDSCs, consisting of more immature progenitors and characterized as Lin^- (including CD3, CD14, CD15, CD19, CD56) $HLA\text{-}DR^-CD33^+$ cells[59, 64]. However, the mouse equivalent is yet to be identified.

Table 1: *Phenotypic characteristics for the identification of Myeloid Derived Suppressor Cells (MDSCs). Table modified from Bronte et al.[59].*

Nomenclature	Mouse Phenotype	Human Phenotype (in PBMC fraction)
Total MDSC	$Gr\text{-}1^+CD11b^+$	Not clearly determined
PMN-MDSC	$CD11b^+Ly6C^{lo}Ly6G^+$	$CD14^-CD11b^+CD15^+$(or $CD66b^+$)
M-MDSC	$CD11b^+Ly6C^{hi}Ly6G^-$	$CD11b^+CD14^+HLA\text{-}DR^{low/-}$ $CD15^-$
eMDSC	Not clearly determined	Lin−(CD3/14/15/19/56)/HLA-DR−/CD33+

Immune suppression is a main feature of MDSCs. Although MDSCs are involved in the suppression of different immune cells, their primary target are the T cells. It has been proposed that MDSCs that infiltrate peripheral lymphoid organs exert their immunosuppressive function via different mechanisms compared to tumor infiltrating MDSCs[25]. In peripheral lymphoid organs, the MDSC-mediated immune suppression is based on antigen specific and contact cell dependent mechanisms as well as the induction of several pathways, including the production of reactive nitrogen and oxygen species[65, 66, 67] [nitic oxide (NO), reactive oxygen species (ROS) and peroxynitrite (PNT)]; depletion of essential amino acids required for T cell proliferation, such as L-arginine via arginase 1 (Arg1)[68], L-cysteine[69] and tryptophan via indoleamine 2,3-dioxygenase (IDO)[70]; decrease of T-cell ability to home to sites where they would be activated by ADAM-17 expression[71]; production and secretion of regulatory cytokines, such as IL-10 and TGF-β[72]; formation of T regulatory cells (Tregs)[73, 74]; and inhibition of NK-cells activity[75]. Evidently, not all of these suppressive mechanisms act simultaneously. After migration from peripheral tissues to the tumor site, MDSCs have been demonstrated to have a more potent nonspecific immunosuppressive activity. At tumor tissue MDSCs are exposed to an inflammatory and hypoxic microenvironment, inducing thus the hypoxia-inducible factor 1-α (HIF1-α), which in turn contributes to the increased expression of inhibitory PD-L1 on the surface of tumor-infiltrating MDSCs[76], upregulation of Arg1[77], elevation of iNOs[77] but downregulation of ROS production[45], and the increased production of CCL4 and CCL5 chemokines resulting in recruitment of Tregs at tumor site[45]. Moreover, MDSCs often mature into TAMs[78] and promote the differentiation of normal fibroblasts to cancer-associated fibroblasts (CAFs)[79]. So, MDSCs are considered to be the cornerstone of the immunosuppressive shield, as they achieve to hide and protect cancer by evading immune surveillance conducted by the patient's immune system.
Extensive studies in recent years have provided adequate evidence of the clinical relevance of MDSCs. MDSCs have been associated with poor prognosis[80, 81], as patients with increased circulating MDSCs double the risk of dying from cancer; augmentation of metastatic process[82]; and increased risk of resistance to immunotherapeutic[83, 84] and chemotherapeutic approaches[85, 86]. In different types of cancer, both peripheral and tumor tissues of cancer patients have been reported for their increased accumulation of MDSCs, including melanoma[87], non-small cell lung cancer[88], lung tumors[89], colon and breast carcinoma[90], renal carcinoma[91], head and neck squamous cell carcinoma[92], prostate cancer[93], pancreatic[94], rectal cancer[95], hepatocellular carcinoma[96] and glioma[97]. Therefore, a better understanding of MDSC biology will result in the designing of successful cancer immunotherapies, through the effective targeting of the molecular mechanisms governing the development and the immune suppressive mechanisms of these cells that lead cancer development and progression.

1.1.2 Dendritic Cells

Dendritic cells (DCs) are differentiated myeloid cells that represent a special group of cells which display different phenotype and activity at the tumor site and exhibit differential pro-tumorigenic and anti-tumorigenic functions. DCs constitute a specialized antigen-presenting cell population, which is present in all tissues, and has a crucial role in the regulation of immune responses[98]. The name of dendritic cells has been corelated with a cell population that is characterized by stellate morphology, increased expression levels of major histocompatibility complex class II molecules (MHC-II) and the expression of the intergrin CD11c[99, 100]. Also, DCs have the ability to migrate from non-lymphoid to lymphoid tissues and prime naive T lymphocytes[101, 102, 103]. Therefore, DCs has a crucial role in inducing and maintaining the antitumor immunity. However, due to the heterogeneity of DC family there are subsets whose function is to suppress rather than stimulate immune responses[104] and thus in the tumor microenvironment have an immunosuppressive and tolerogenic role, which limit activity of effector T cells and support tumor growth and progression.
Both human and murine DCs, irrespective of their primary location in secondary lymphoid organs or in the parenchyma of non-lymphoid organ, can be distinguished into four distinct subsets based on the phenotypic markers and functional characteristics that they share: (a) $CD8\alpha^+$ or $CD103^+$ DC-like cells (conventional DC1s), (b) $CD11b^+$ DC-like cells (conventional DC2s), (c) $CD11b^+Ly6C^+$ monocyte-derived DCs (Mo-DCs) and (d) plasmacytoid DCs (pDCs)[105] (**Table 2**). The main characteristic of all these subsets and DCs generally is their maturation process in the presence of stimuli, including pathogen- or a danger-associated molecular patterns. Additionally, the cancer cell-mediated secretion of immunosuppressive factors has been accused of being involved in the regulation of DC differentiation, maturation and function[106, 107]. This kind of activation and maturation of DCs, which is occurred mainly via the inflammasome central signaling axis of the immune system, is immunogenic owing to the up-regulation of MHC class II and co-stimulatory molecules as well as secretion of proinflammatory cytokines, such as IL-1β, IL-12, TNF-α and IL-6[108, 109]. Conversely, immature or semi-mature DCs have been demonstrated to induce T-cell tolerance via deletion, anergy or induction of Tregs, as DCs that express elevated levels of MHC class II and co-

stimulatory molecules, whereas do not secrete IL-1β, IL-6, TNF-α and IL-12 have been reported to display tolerogenic rather than immunogenic and stimulatory phenotype[109, 110].

***Table 2**: Phenotypic characteristics for the identification of Dendritic cells (DCs). Table modified from Jahchan et al.[111].*

Nomenclature	Mouse Phenotype	Human Phenotype (in PBMC fraction)
cDC1	$XCR1^+CLEC9A^+FLT3^+MHC\text{-}II^+CD11c^+CD11b^-CD24^{hi}CD103^{+/-}CD8a^{+/-}$	$BDCA3\ (CD141)^+XCR1^+CLE9A^+HLA\text{-}DR^+CD14^+$
cDC2	$SIRPA^+CD11c^+FLT3^+MHC\text{-}II^+CD11b^{+/-}$	$BDCA1\ (CD1c^+)HLA\text{-}DR^+$
pDC	$B220^+Ly6C^+BST2^+SinglecH^+CD11c^{lo}$	$Lineage^-HLA\text{-}DR^+BCCA2^+BDCA4^+CD123^+CD45RA^+CD33^-$
Mo-DC	$CD11b^+CD11c^+SIRP\alpha^+CD64^+MHC\text{-}II^+F4/80^+Fc\varepsilon RI^+Ly6C^+$	$BDCA1^+HLA\text{-}DR^+CD14^+CD1A^+\ Fc\varepsilon RI^+CD206^+CD64^+$

Despite the fact that the presence of mature DCs in tumors has been associated with a good prognosis in several types of cancer[112, 113], clinical data have shown defective functionality and scarcity of mature DCs intratumorally[106, 114, 115]. Moreover, the switch of DCs from an immunostimulatory activation state, which drives anti-tumor immune responses, to an immunosuppressive activation state, has also been reported at different stage of tumor development[116]. Tumor-associated DCs (TADCs) seems to be guided by the tumor to maintain immune tolerance or immunosuppression[117], as in a murine breast cancer model tumor infiltrated DCs, in spite of the fact that they present tumor antigens in tumor specific T cell they fail to fully activate and stimulate them effectively[118]. Moreover, the immunosuppressive role of TADCs is revealed by the upregulation of inhibitory molecules such as B7-H1[119], by the activation of arginase[120], by the induction of indoleamine 2,3-dioxygenase (IDO)[121], or the activation of oxygen oxygen-dependent pathways, which downregulate CD3 and lead T cells to apoptosis[122]. Except from immunosuppression, TADCs seems to have a role in tumor development and progression as they favor neovascularization and induce cancerous cell growth and spreading[106, 117, 123].
This dendritic cell dichotomy, may be is attributed to tumor microenvironmental signaling that might promote the differentiation of specific TADC subsets[124] with tolerogenic and immunosuppressive potential, which are responsible for antigen-specific unresponsiveness in the lymphoid organs, in the periphery and at tumor site[123, 125]. For example, mo-DCs or also called inflammatory DCs, which are transiently formed in response to microbial or inflammatory stimuli and disappear once the inflammation resolves and are characterized by the expression of Ly6C, CD11b, MHC-II and CD11c, might exhibit immunosuppressive properties under certain circumstances as they are prominent in tumor antigen uptake, but lack strong T-cell stimulatory capacity due to NO-mediated immunosuppression[109, 126]. Furthermore, pDCs have been demonstrated to induce tolerance and to be correlated with poor outcome in cancer types such as breast and ovarian cancer[127]. Despite this, some studies have shown their potent anti-tumor activity by efficiently presenting antigens to cytotoxic T lymphocytes, inducing thus immune responses[127, 128]. Thus, a wide range of cells and factors in the tumor microenvironment represent an example of the differential stimuli that affect the DC maturation and differentiation into several subsets that either promote or prevent anti-tumor immunity. Therefore, a better understanding of basic TADC biology and molecular mechanisms that control functionality and longevity of DCs will be essential for envisaging targeted immunotherapies.

1.2 Inflammasome

1.2.1 An overview

The first line of defense and the gatekeeper in the defense system is the innate immune system. Innate immune is activated and responds to any pathogens and exogenous stimuli, and these responses are based on pattern recognition receptors (PRRs) and the effects of the downstream signaling which activate. PRRs were originally identified as elements of the immune cells and mainly of the innate immune cells. Thus, the expression of these receptors is documented on the cells at the front line of defense, such as monocytes, dendritic cells (DCs), macrophages, neutrophils and epithelial cells, but also cells that belong to the adaptive immune system[129, 130, 131].
The PRR family is composed of various types of extracellular and intracellular receptors and until now six classes of PRRs have been identified, including Toll-like receptors (TLRs)[132], the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs)[133], the nucleotide-binding oligomerization domain-like receptors (NLRs)[134, 135, 136], the Scavenger receptors[137], the C-type lectin receptors (CLRs)[138], and the absent-in-melanoma (AIM)-like receptors (ALRs)[139, 140].

PRRs are able to recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), flagellin, lipoproteins, but also bacterial, viral and fungal RNA, DNA and structural proteins[141, 142]. Except from PAMPs, they can also detect host-derived danger signals, termed damage-associated molecular patterns (DAMPs), which are endogenous substances released from damaged and dying cells in the extracellular compartment upon cellular stress, apoptotic or necrotic death resulting in their accumulation at inflamed and tumor sites[142, 143]. Such molecules are oxidized and/or methylated DNA, high-mobility group box 1 (HMGB1), reactive oxygen species (ROS), heat-shock proteins, mitochondrial DNA and adenosine triphosphate (ATP)[144, 145, 146]. Then, DAMPs/PAMPs recognition leads to the general activation of the immune cell and the expression of pro-inflammatory genes.
Among the six classes of PRRs, the class of NLRs, predominantly expressed by cells of the myeloid lineage, are one of the most well-studied[147]. NLRs are exclusively cytoplasmic sensors and trigger a cascade of downstream responses. The human NLR gene family is comprised of 22 members and at least 34 members in the mouse, and their proteins are characterized by three major domains: a central conserved nucleotide-binding and oligomerization domain (NACHT), which is flanked by a carboxy-terminal ligand-binding region consisted of leucine-rich repeats (LRRs) and an amino-terminal effector domain responsible for protein–protein interactions for downstream signaling[129, 147, 148]. Of these three domains, only the NACHT domain is common to all members of the NLR family and is needed for the regulation of the NLRs' ATP-dependent self-oligomerization. The nature of the amino-terminal effector domain determines the four sub-groubs of the NLR family and include adaptor proteins with an acidic transactivation domain (NLRA proteins), a pyrin domain (PYD) (NLRP proteins), a caspase activation and recruitment domain (CARD) (NLRC proteins) or a baculovirus inhibitor of apoptosis repeat domain (BIR) (NLRB proteins). The carboxy-terminal region contains multiple LLR elements that are responsible for ligand sensing, thus determining the specificity of each ligand based on the types of NLRs[149, 150]. The interaction each one of the NLRs with a specific PAMP/DAMP leads to the NLR oligomerization to protein complexes, which are called inflammasomes.
A central signaling axis of the immune system that regulates and control the homeostatic innate immune pathways, is considered to be the inflammasomes[151]. Inflammasomes, reported and identified for the first time by Jurg Tschopp in 2002[152], are cytoplasmic macromolecular complexes consisting of several proteins, that trigger and orchestrate central inflammatory responses in immune cells and operate as a platform for the activation of cysteine protease caspase-1, resulting in the maturation and secretion of the proinflammatory cytokines and in the induction of the pyroptotic cell death[129, 135]. The inflammasome constitutes an innate immune sensor against pathogen-associated or endogenous danger-associated signals. Up to date, there are six well-established inflammasomes that have been described: the NLR-family, PYD-containing 1 (NLRP1); the NLR-family, PYD-containing 3 (NLRP3); the NLR-family, CARD-containing 4 (NLRC4); the NLR-family, apoptosis inhibitory protein (NAIP); the absent in melanoma 2 (AIM2) and the pyrin inflammasomes[153]. In addition, they have also been described other members of the NLR family that have the potential to form inflammasome complexes, such as NLRP2[154], NLRP6[155], NLRP7[156], NLRP9[157], and NLRP12[158]. However, except from their protecting functions in the host defense system, the overactivation of inflammasome complexes has been associated with many pathophysiological conditions, including autoinflammatory diseases, such as cryopyrin-associated periodic syndromes (CAPS) and familial Mediterranean fever (FMF), and excessive inflammation, which among the others is correlated with cancer development.

1.2.2 NLRP3 Inflammasome

Biological and Functional characteristics. Between all the aforementioned inflammasome complexes, the NLRP3 inflammasome has been extensively investigated due to its possible association with a number of human pathogenic conditions. However, many of the functions of the NLRP3 inflammasome and its effects on human diseases remain unclear. The NLRP3 protein, which is also termed as cryopyrin or NALP3, have been reported to predominately be expressed in lymphoid organs and organs flooded by immune cells[159]. A study by the team of Jürg Tschopp in 2011[160], documented the elevated expression of NLRP3 upon exposure to inflammatory stimuli primarily by myeloid cells, such as splenic neutrophils, macrophages, monocytes and conventional dendritic cells, whereas NLRP3 was barely expressed by lymphoid subsets (splenic lymphoid B, T, and NK cells), eosinophils and plasmacytoid dendritic cells. Since then, NLRP3 expression and its downstream signaling responses have been extensively investigated in macrophages, in many human diseases, and in particular in many types of cancer[161, 162, 163, 164, 165, 166]. In addition, NLRP3 inflammasome has been also studied in other cell types, like dendritic cells[167, 168, 169], fibroblasts[170, 171, 172], epithelial cells[171, 173, 174], myeloid-derived suppressor cells (MDSCs)[43, 175, 176, 177, 178]. Despite the barely NLRP3 expression in lymphoid subsets reported by Jürg Tschopp in 2011, there are both previous and

recent reports of NLRP3 expression in B and T lymphocytes[159, 179, 180, 181]. Thus, despite the extensive research done around the NLRP3 inflammasome, many is yet undocumented and have to be revealed about its expression profile and role in several diseases, and particular in cancer.

Regarding the structural characteristic of NRLP3 inflammasome, is a protein complex that includes the NLRP3 sensor/receptor, the apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein, which is a bipartite molecule containing an amino-terminal PYD domain and a carboxy-terminal CARD domain, and the caspase-1 effector protein[153]. The NLRP3 sensor protein, as already mentioned about the NLRP proteins, has a tripartite structure consisting of an amino-terminal PYD domain, a nucleotide-binding and oligomerization NACHT domain, and a carboxy-terminal LRR domain. Upon stimulation and inflammasome activation, NLRP3 sensor protein oligomerizes through protein-protein interactions between NACHT domains. Then, NLRP3 oligomerization leads to the recruitment of ASC adaptor protein through homotypic PYD–PYD interactions and the formation of a macromolecular helical structure, termed as ASC speck, composed by ASC filamentous oligomers and occurred through PYD–PYD interactions[182, 183]. The ASC speck formation follows the recruitment of caspase 1 effector protein, through association of ASC CARD domain with caspase 1 CARD domain, resulting in caspase 1 self-cleavage and activation, with the proteolytically active part of caspase 1 to remain bound to ASC[184]. Last, another component that appears to be essential for NLRP3 inflammasome activation and caspase 1 maturation is a serine-threonine kinase, called NIMA-related kinase 7 (NEK7), which exclusively interacts with NLRP3 and no other inflammasome sensors and through its oligomerization with NLRP3 forms a complex needed for ASC speck formation and caspase 1 maturation[185, 186, 187]. Thus, the multiprotein NLRP3 inflammasome complex is formed and with caspase 1 activation, the processing of the immature pro-forms of pro-inflammatory IL-1β and IL-18 cytokines into their mature and biologically active forms occurs.

Despite the fact that both IL-1β and IL-18 are pro-inflammatory cytokines belonging to the IL-1 cytokine family, their biological role is completely different, with also contrasting properties in the tumor milieu. IL-1β is a potent proinflammatory mediator in many immune responses, whose secretion induces the expression of pro-inflammatory genes, such as IL-6 and TNF-α, associated with acute phase reactions, including fever, vasodilatation, pain threshold, hypotension, the infiltration of innate immune cells to the site of inflammation and the modulation of the cells of adaptive immunity[188, 189]. From the perspective of cancer, IL-1β can have both pro- and anti-tumorigenic role. The IL-1β- dependent IL-6 and TNF-α induction exert their pro-tumorigenic activity, the first through the activation of STAT-3[190] and the latter through the infiltration of immune-suppressor cells that promote tumor development and progression, such MDSCs[191]. However, several times the IL-1β- dependent TNF-α expression can have anti-tumorigenic effects[192]. On the other hand, IL-18 is not associated with a febrile response and is necessary for the production of interferon-gamma (IFN-γ) by T cells, such as $CD4^+$ and $CD8^+$ cells, and natural killer (NK) cells, thus constituting a co-stimulatory cytokine that induces Th1 cell responses and potentiates the cytolytic activity of the immune cells[189, 193]. This Th1 polarization and cytolytic activity of NK cells is mediated by the simultaneous presence of IL-18 and IL-12, whereas the absence of the IL-12 cytokine can promote a Th2 response[194]. Therefore, depending on the tumor microenvironment, these two cytokines can have divergent roles in the control of tumor-associated inflammation and tumor surveillance. Both cytokines have the ability to suppress or promote the carcinogenesis, but the important determinants for these processes are the type, the stage and the contexture of the tumor.

Apart from the maturation and secretion of these two pro-inflammatory cytokines, the activation of NLRP3 inflammasome can result in pyroptosis. Pyroptosis, is an inflammatory cell death that promotes the inflammation and is characterized by membrane rupture and the release of pro-inflammatory molecules to the extracellular compartment, such as mature forms of IL-1β, IL-18, IL-1α and high-mobility group box 1 (HMGB1), which are recognized by the neighbor cells as DAMPs and the immune system responses are further induced. The pyroptotic cell death, is characterized by the formation of membrane pores, which are the outcome of the caspase 1-mediated proteolytic maturation of the pore-forming protein gasdermin D (GSDMD). These maturation process results in Gasdermin N-terminal fragment, which then polymerizes, the pyroptotic pore is formed and thus the cell death occurs[195, 196, 197, 198].

The generation of a functional NLRP3 inflammasome is based on a two-step mechanism, which requires both a priming and an activation step, with the latter being accomplished through three different signaling pathways: the canonical, the noncanonical, and the alternative inflammasome pathways[199, 200, 201].

Priming. Priming is a critical step for NLRP3 inflammasome activation and this is made clear by a study reported that treatment of mouse bone marrow-derived macrophages with NLRP3 activators alone have no effect on the

activation of the NLRP3 inflammasome, whereas the pretreatment with a signal that indicates the presence of infection, such as Lipopolysaccharides (LPS), induced the NLRP3 inflammasome activation[202]. This kind of stimulation is required, as previously described, for the binding to the TLRs, NLRs, or cytokine receptors and thus activating the NF-κB-dependent transcriptional pathway (**Figure 2**). Therefore, priming step is essential for two functions required for the inflammasome activation. The first function, as already understood, is associated with the upregulation of the NLRP3 inflammasome components, including the NLRP3 and pro-IL-1β. Macrophages in resting and steady state condition are documented to have insufficient amounts of NLRP3 for the induction of inflammasome activation[153, 202]. So, a wide range of inflammasome triggers, such as PAMPs and DAMPs, are involved in the Nlrp3 transcriptional priming. This transcriptional upregulation is NF-κB-dependent and follows the inflammatory signaling started from TLRs and the recruitment of their adapter proteins, including myeloid differentiation primary response gene 88 (Myd88) and Toll-interleukin receptor domain-containing adapter protein inducing IFNβ (TRIF), and followed by the IRAK (interleukin-1 receptor-associated kinases)-dependent phosphorylation events[202, 203, 204, 205]. In addition, recent studies documented that NF-κB-dependent Nlrp3 transcription during the priming process is also regulated by downstream signaling molecules of the TNF receptor, including FAS-associated death domain protein (FADD) and caspase-8[202, 206, 207, 208]. Like the NRLP3, IL-1β, which is mainly expressed in myeloid cells[209, 210], is not constitutively expressed at resting state but in a TLR-dependent inducible manner[202, 211]. The IL-1β transcriptional upregulation is, like the Nlrp3 transcription, NF-κB-dependent through a similar signaling pathway, which is described in more detail below (see IL-1β synthesis). Despite the NLRP3 and pro-IL-1β, the expression levels of ASC[202], pro-caspase-1[43, 202], and pro-IL-18[212, 213] do not appear to be inducible by the presence of a priming signal and are constitutively expressed. However, some reports suggest that both caspase-1 and IL-18 may also be upregulated, the first by the NF-κB or Interferon regulatory factor (IRF)-1[214], and the latter by interferon priming via STAT1/2 and IRF-9[212, 213, 215].

The second function of priming, is related to the post-translational modifications of NLRP3[216]. This non-transcriptional role of priming is very critical for the function and activity of the NLRP3 protein, as wells as for its stability. Post-translational modifications, including ubiquitylation, phosphorylation and sumoylation, stabilize the NLRP3 protein in an inactive but signal-competent configuration and prepare it so that be ready to promptly respond to an activating signal, allowing thus its oligomerization[199, 217]. However, despite the transcriptional role of priming, several studies have suggested that priming and its post-translational modifications, regulate the NLRP3 inflammasome activation in transcription-independent way. In more detail, the signaling molecules TRIF and IRAK1 promotes the NLRP3 inflammasome activation independently of the IKK complex and thus the NF-kB pathway, underlining the role of priming in the post-translational regulation[202, 218, 219]. Furthermore, the NLRP3 protein is ubiquitylated in macrophages at steady state and upon priming signals, such as LPS, is deubiquitylated by Lys-63-specific deubiquitinase BRCC36 (BRCC3), and thus NLRP3 activation is promoted in the absence of NLRP3 activator[220, 221, 222, 223]. These evidences indicate that it is not necessary for the activation of the NLRP3 inflammasome to be the result of a transcriptional upregulation through the priming step, but the priming step can be transcription-independent and signal the NLRP3 inflammasome, by post-translational modifications, leading to its active form. In addition, a recent study proposed a mechanism, in which the TLR4-dependent priming signals results in the IRF1-mediated synthesis of mitochondrial DNA (mtDNA) that acts as NLRP3 inflammasome activator[224]. Therefore, the priming step is vital for the inflammasome activation, as through transcription-dependent and -independent pathways the NLRP3 inflammasome activation could be achieved

Activation. The priming step prepares the inflammasome components for the activation, whereas the second step, called activation step, is followed by the recognition of the NLRP3 inflammasome activator and thus the induction of the full NLRP3 inflammasome activation and formation. NLRP3 inflammasome can be activated by a wide variety of activating stimuli that trigger the specific activation of NLRP3, the assembly of a functional inflammasome complex, and last the maturation of caspase-1 enzyme. The NLR3 activators include exogenous stimuli, such as bacterial, viral, fungal infections and environmental irritants, as well as endogenous molecules, among which are adenosine triphosphate (ATP), amyloid-β, monosodium urate (MSU), and cholesterol crystals (**Table 3**). The unifying feature of these NLRP3 activators is that they all lead to the induction of cellular stress, which in turn is sensed by the NLPP3 sensor, as the physical interaction between the NLRP3 and its activator is unlikely due the wide chemical and structural diversity of the NLRP3-activating molecules. The exact way that the NLRP3 senses this stress response from each NLRP3 activator and the exact molecular and cellular events that are induced for NLRP3 assembly and activation differ between the varying stimuli and are not fully elucidated. However, there are six main upstream signals, which may act together or independently, through which the different PAMPs and DAMPs stimuli trigger

the activation and assembly of the NLRP3 inflammasome: a) ionic flux events, such as potassium ions (K^+) efflux, calcium ions (Ca^{2+}) mobilization, chloride ions (Cl^-) efflux and sodium ion (Na^+) influx, b) lysosomal rupture, c) mitochondrial dysfunction, d) reactive oxygen species (ROS) generation, e) metabolic changes, f) trans-Golgi disassembly[199, 201, 217, 225]. Although, there are data suggesting that many of these signaling pathways are interrelated and overlapping, and thus there is not an unambiguous model for NLRP3 activation.

Table 3: NLRP3 inflammasome activators. *The NLRP3 inflammasome is activated by specific exogenous (PAMPs) and endogenous (DAMPs) stimuli. Table modified from Swanson et al.[199].*

Activator	Source	Examples
DAMP	Self-Derived	ATP, cholesterol crystals, monosodium urate crystals, calcium pyrophosphate dihydrate crystals, calcium oxalate crystals, soluble uric acid, neutrophil extracellular traps, cathelicidin, α-synuclein, amyloid-β, serum amyloid A, prion protein, biglycan, hyaluronan, islet amyloid polypeptide, hydroxyapatite, haeme, oxidized mitochondrial DNA, membrane attack complex, cyclic GMP–AMP, lysophosphatidylcholine, ceramides, oxidized phospholipid 1-palmitoyl- 2-arachidonoyl-sn-glycero-3-phosphorylcholine and sphingosine
	Foreign-Derived	Alum, silica, aluminium hydroxide, nanoparticles, carbon nanotubes, chitosan, palmitate (also self-derived), UVB, imiquimod (R837)/CL097 and resiquimod (R848)
PAMP	Bacterial	Lipopolysaccharide, peptidoglycan, muramyl dipeptide, trehalose-6,6'-dibehenate, c-di-GMP–c-di-AMP, bacterialRNAandRNA–DNAhybrid
		Toxins: nigericin (Streptomyces hygroscopicus), gramicidin (Brevibacillus brevis), valinomycin (Streptomyces fulvissimus and Streptomyces tsusimaensis), β-haemolysin (Streptococcus sp. 'group B'), α-haemolysin (Staphylococcus aureus), M protein (Streptococcus sp. 'group A'), leucocidin (Staphylococcus aureus), tetanolysin O (Clostridium tetani), pneumolysin (Streptococcus pneumoniae), listeriolysin O (Listeria monocytogenes), aerolysin (Aeromonas hydrophila), streptolysin O (Streptococcus pyogenes), enterohaemolysin (Escherichia coli O157:H7), haemolysin BL (Bacillus cereus), adenylate cyclase toxin (Bordetella pertussis), M protein (Streptococcus sp. 'group A') and maitotoxin (Marina spp. dinoflagellates)
	Viral	Double-stranded RNA and single-stranded RNA
	Fungal	β-Glucans, hyphae, mannan and zymosan

K^+ efflux is the most common ionic event induced in response to NLRP3 activator, including extracellular ATP and nigericin, and decrease of the cytosolic K^+ concentration has been reported to mediate IL-1β maturation and secretion, and thus NLRP3 inflammasome activation[226, 227], whereas high extracellular K^+ concentrations prevent the activation process[228, 229]. Extracellular ATP, is one of the most common NLRP3 activator used in many in NLRP3 inflammasome activation assays. After binding of ATP to its cell surface receptor, the P2X purinoceptor 7 ($P2X_7$), it was believed that NLRP3 activation is mediated only through the K^+ efflux[226, 230]. However, the $P2X_7$ receptor is a non-selective cation channel for Na^+, K^+ and Ca^{2+} [231]. Thus, the ATP stimulation induce the $P2X_7$-mediated Ca^{2+} and Na^+ influx and $P2X_7$ in turn coordinates with the TWIK2 K^+ channel, which mediates the efflux of K^+ ions resulting in NLRP3 inflammasome assembly and activation[232]. Similar to ATP, pore-forming toxins, such as the bacterial toxin nigericin as well as silica, alum and calcium pyrophosphate crystals all aim to the decrease of cytosolic K^+ concentration that is vital for NLRP3 inflammasome activation[228]. Despite the extensive research for the K^+ efflux-mediated activation, the mechanistic link for the way that K^+ concentration alterations affect the NLRP3 activation is not completely understood, but is suspected that intracellular K^+ concentration may affect the NLRP3 conformation[233].

Due to the implication of NLRP3 inflammasome in many pathogenic conditions, the research interest have focused on the identification of small molecule compounds that target the NLRP3 and inhibit its assembly and activation. Several such molecules have been documented up to date, including parthenolide[234], 3,4-methylenedioxy-β-nitrostyrene[235], dimethyl sulfoxide (DMSO)[236], type I IFN[237] and other inhibitory compounds[199]. Nevertheless, some of these compounds are non-specific and have limited potency, while others should be further investigated about their effects. In 2015 a study by Coll et al. reported a diaryl-sulfonylurea-containing compound and one of the most promising molecules, called MCC950 or otherwise known as CP-456,773[238]. This study revealed the specificity of the MCC950 compound to selectively inhibit NLRP3 inflammasome activation and its effectiveness in mouse disease models of inflammation and autoimmunity *in vivo*. Thus, MCC950 agent has the ability to specifically inhibit the activation of both the canonical and the noncanonical NLRP3 inflammasomes and the secretion of IL-1β in mouse and human macrophages, while it does not affect and impairs the activation of AIM2, NLRC4, or NLRP1 inflammasome[238, 239, 240]. At a molecular level, MCC950 inhibitor prevents the activation by inhibiting the NLRP3-iduced ASC speck oligomerization[238], and ASC complexes, as it has already mentioned, act as protein scaffolds leading to the activation of caspase-1 and the subsequent maturation of IL-1β and IL-18. Furthermore, this MCC950-mediated inhibition is independent of the TLR signaling inhibition, inhibition of the priming step of NLRP3 activation,

potassium and calcium fluxes or protein-protein interactions between NLRP3–ASC and NLRP3–NLRP3[238]. Two recent studies[241, 242] shed light to the inhibition mechanism of MCC950 inhibitor, reporting that MCC950 reversibly binds to the ATP-binding region and specifically to Walker B motif leading to the inhibition of ATP hydrolysis[241] and the preservation of a closed, inactive NLRP3 conformation or its enforcement if the NLRP3 is already in an active, open state[242]. The ATP-hydrolysis region is located in the NACHT domain of NLRP3 and contains Walker A and Walker B motifs associated with the binding and hydrolysis of ATP, respectively, and is crucial for the NLRP3 activation[243]. MCC950 inhibitor has been used and tested of its therapeutic effects in a wide range of immunopathological models, such as CAPS[238], experimental autoimmune encephalomyelitis[238], rheumatoid arthritis[244], diabetes[245, 246, 247], Alzheimer disease[240], Parkinson's disease[248], dermal and pulmonary inflammation[249], traumatic brain injury[250], ischemic stroke[251], myocardial infarction[252], atherosclerosis[253], cardiac arrhythmias[254], steatohepatitis[255, 256], nephropathy[257, 258] and colitis[161, 259]. In addition, despite the widespread use of MCC950 inhibitor at blocking pathological effects of NLRP3 in various diseases, its therapeutic efficacy in cancer treatment has not been studied at all[8], with the only reports of its therapeutic effect being at head and neck squamous cell carcinoma[260] and at a melanoma model[261]. Thus, the idea of tumor immunoediting by targeting NLRP3 inflammasome signaling, opens new avenues in anticancer therapy.

Non-canonical NLRP3 Inflammasome. Except from the classical/canonical NLRP3 activation pathway, there is also an another inflammasome pathway, called non-canonical NLRP3 Inflammasome (**Figure 3**), which represents an additional level of cellular defense and responds to pathogens, especially to Gram-negative and not to Gram-positive bacteria, that bypass the cell membrane and cell surface TLR4 receptor, ending up in the cytosol[262]. It has been reported that non-canonical NLRP3 inflammasome is activated by the detection of cytoplasmic LPS delivered into the cytosol either by transfection or infection by Gram-negative bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, *Citrobacter rodentium*, *Shigella flexneri*, and *Burkholderia thailandensis*, and this activation is independent of TLR4 signaling[263, 264, 265]. The non-canonical NLRP3 activation process involves signaling through the

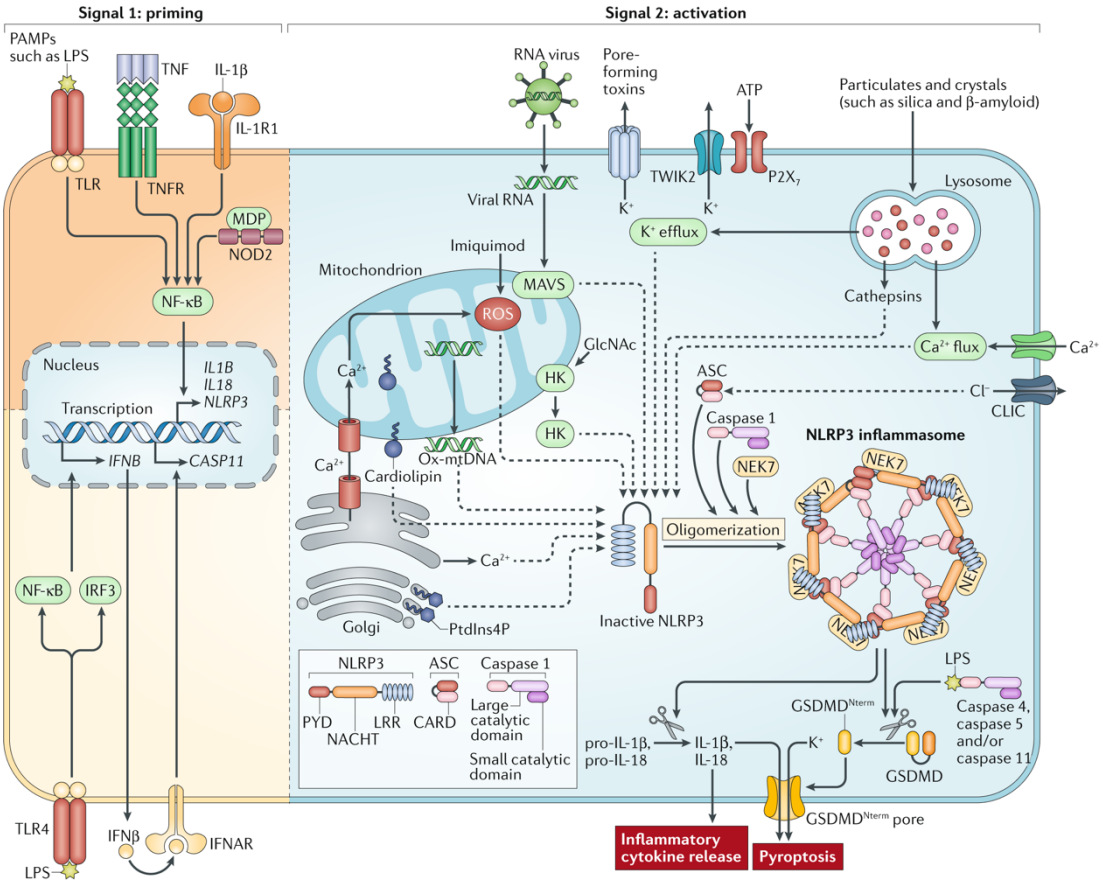

Figure 2: Mechanisms involved in priming and activation of NLRP3 canonical pathway. *NLRP3 inflammasome activation requires two signals. The signal 1 (priming) is provided by the activation of cytokines (e.g. TNF-α, IL-1ß) or PAMPs that are recognized by PRRs (e.g. TLRs), leading to the transcriptional upregulation of canonical NLRP3. The signal 2 (activation) is provided by any of numerous PAMPs or DAMPs (Table 3), that activate multiple upstream signaling events. The figure is adapted from Swanson et al.[199].*

caspase-11 in mice and caspases-4/5 in humans, in contrast to canonical NLRP3 inflammasome that requires caspase-1[266, 267]. As in the canonical pathway, the priming is very crucial for the expansion of the inflammatory response through the transcriptional activation of inflammasome components. Thus, in the non-canonical pathway extracellular LPS binds in the TLR4 receptor and through the TRIF and MyD88 signaling, type I IFNs are induced and, together with the complement C3-C3aR axis, upregulate the expression of caspase-11[268], Guanylate-Binding Proteins (GBPs)[269] and Immunity Related GTPase family member 10 (IRGB10)[270]. GBPs and IRGB10 are GTPases that are recruited to the intracellular bacteria and lyse them, resulting in liberation of LPS in the cytosol[269, 270]. Then, the cytosolic LPS is bound directly on caspase-11/4/5 CARD domains, but not other caspases, and triggers their oligomerization and activation through their auto-proteolytic cleavage[271, 272]. Caspase-11/4/5, like caspase-1, also enzymatically cleave gasdermin D (GSDMD) inducing pyroptosis, by the formation of membrane pores and thus the membrane disruption[197, 198]. In addition, activated caspases also cleave the cytosolic region of pannexin-1 protein, a membrane channel that transfers the intracellular ATP to the extracellular compartment, which in turn activates the ATP-selective channel P2X7R[197, 273, 274]. Both the activation of P2X7R ATP channel and GSDMD contribute to pyroptosis and the induction of K^+ efflux that activates canonical NLRP3 inflammasome pathway and thus the secretion of mature IL-1β and IL-18. Therefore, caspases 11, 4 and 5 of non-canonical inflammasome are not responsible for the maturation cleavage and the secretion of IL-1β and IL-18, but through the induction of the non-canonical NLRP3 inflammasome the pyroptosis is triggered, which in turn favors the activation of the canonical NLRP3 inflammasome and the subsequent cytokine release.

Alternative NLRP3 Inflammasome. Despite the canonical and non-canonical NLRP3 inflammasome pathways that are based on a two-step activation model and require a secondary signal, there is another NLRP3 inflammasome pathway that follow one-step activation model and is referred as alternative NLRP3 inflammasome (**Figure 3**). Alternative NLRP3 inflammasome has been reported in human and porcine monocytes but not in murine monocytes[275, 276]. Human monocytes, except from the LPS stimulation, do not need a secondary stimulus, so to activate the caspase-1 and thus the induction of cytokine maturation and secretion. LPS is recognized by TLR4, which in turn triggers an intracellular signaling cascade via the TRIF, RIPK1, FADD and caspase-8 proteins, resulting in caspase-8 catalytic activity upstream of the NLRP3. The activation of caspase-8 leads to the activation of NLRP3 inflammasome and the maturation of IL-1β and IL-18. Additionally, in this alternative inflammasome activation ASC and caspase-1 are also required. However, in spite of both canonical and non-canonical NLRP3 inflammasome pathways, the alternative route is not associated with ASC speck formation, K^+ efflux and pyroptosis and thus the IL-1β secretion is independent of the gasdermin D (GSDMD) pore formation[275].

Furthermore, the one-step activation model has not been observed only in human monocytes but also in mouse bone marrow-derived DCs and mouse splenic DCs, which can also secrete mature IL-1β in response to TLR ligands alone, such as LPS, and in a NLRP3-dependent manner[169]. In adittion, the NLRP3-mediated IL-1β production is ATP-P2X7R independent, as pharmacological inhibition and genetic ablation of P2X7R did not affect LPS-induced IL-1β secretion in murine DCs, both *in vitro* and *in vivo*. This one-step DCs' rapid response might contribute as a rapid defense mechanism against the bacterial septic shock by LPS administration.

Moreover, despite the fact that the vast majority of the studies propose that the macrophages' stimulation with TLR ligands alone is not sufficient to induce Nlpr3 inflammasome activation, here comes a study to report the one-step activation pathway. In mouse bone marrow-derived macrophages (BMDM), the stimulation of different TLRs (TLR2, TLR4, TLR7 and TLR9) with their ligands and the simultaneous stimulation of NLRP3 inflammasome with extracellular ATP, results in a rapid activation of NLRP3 inflammasome that is independent of de novo gene transcription[219]. However, this inflammasome activation does not lead to an IL-1β secretion and to the pyroptotic cell death, but is results in IL-18 secretion. This rapid NLRP3 inflammasome pathway, which is transcription independent, gives the ability to the host to respond rapidly against the invading pathogen that trigger this response, so to decrease the pathogen burden and then the transcriptional induction of inflammatory cytokines and chemokines will occur to further enhance the immune response.

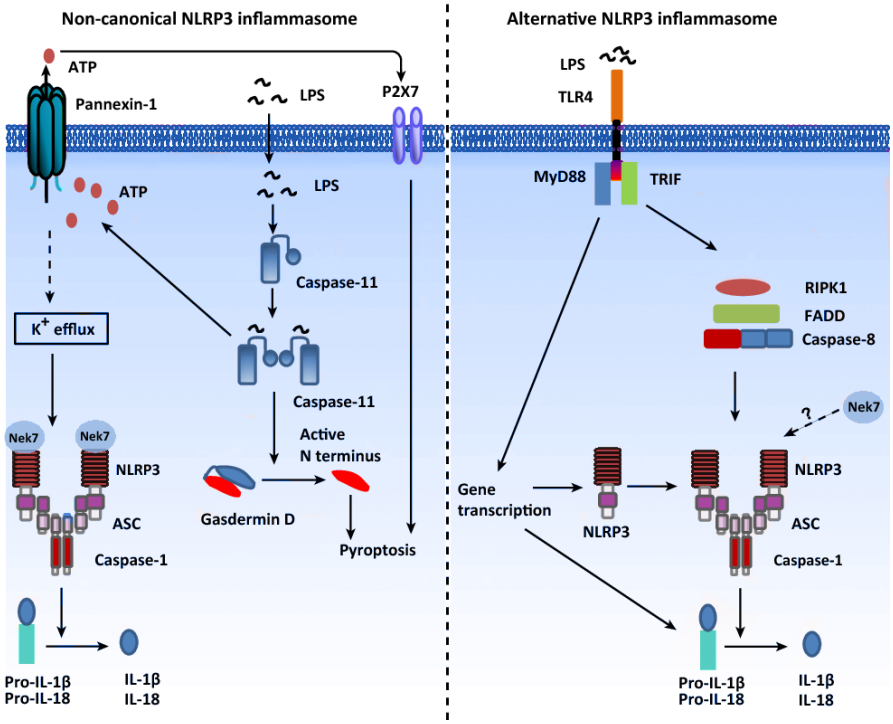

Figure 3: *Mechanisms of activation for Noncanonical and Alternative NLRP3 Inflammasomes. The figure is adapted from He et al.*[201].

1.3 IL-1β

1.3.1 Introduction to the IL-1 family of cytokines

IL-1β is one of the most well-studied members of the IL-1 cytokine family and is involved in many physiological and pathophysiological conditions, including cancer. The members of IL-1 family have both pro-inflammatory and anti-inflammatory properties and their categorization is based on shared receptor or co-receptor binding, and are divided in three subfamilies: the IL-1 subfamily, the IL-18 subfamily and the IL-36 subfamily. The first subfamily, in which IL-1β belongs, is IL-1 subfamily and consists of IL-1α, IL-1β and IL-33 and bind the co-receptor IL-1R3. This subfamily consists of cytokines that signal pro-inflammatory events through the production of other cytokines and chemokines. IL-18 and IL-37 comprise the second subfamily, the IL-18 subfamily, which bind IL-1R5 (also known as IL-18Rα) and induce both pro-inflammatory and anti-inflammatory signaling pathways. In particular, IL-18 binds to the co-receptor IL-1R7 and this binding has as a result the induction of pro-inflammatory signaling pathways, whereas the other member of this subfamily, the IL-37, through its binding to the IL-1R8 co-receptor exerts its anti-inflammatory effects. Lastly, the IL-36α, IL-36β, IL-36γ and IL-38 cytokines form the IL-36 subfamily which share IL-1R6 (also known as IL-36R) binding. In this subfamily, all the IL-36 cytokines (IL-36α, IL-36β and IL-36γ) promote pro-inflammatory effects, while the IL-38, as IL-37, is an anti-inflammatory cytokine[277, 278].

Each member of the three above subfamilies exist as a pro-form, called pro-peptides or pro-cytokines, that reside in the cytosol of the immune cells and under proteolytic maturation are cleaved to generate the biologically active, mature cytokine that can be secreted and bind to the corresponding receptors. This proteolytic cleavage is based on a conserved three amino acid consensus sequence AXD that exists in each member of the IL-1 family. The A in this consensus sequence indicates an aliphatic amino acid, X represents any amino acid, while D is aspartate[279]. Nine amino acids downstream the consensus sequence takes place the N-terminus cleavage site, in which happens the maturation step and the conversion of the pro-form into the bioactive form, thus enabling the appropriate folding of each cytokine for receptor binding and biological activity[279].

IL-1β is considered to be the best characterized and most studied of all the IL-1 family members. IL-1β is a potent pro-inflammatory cytokine with a critical role in the regulation of host-defense responses to infection and injury and also acts as an amplifier of immune reactions[280]. For a quite a long time, IL-1β was supposed to be important only for the induction of the innate immune responses and consequently for the shaping of the adaptive immunity, resulting in resolving of acute inflammations[277]. However, findings come to demonstrate that gain-of-function

mutations in inflammasome components exhibit excessive IL-1β production, which is associated with pathologic conditions, including autoimmune[281] and autoinflammatory[280] diseases, challenging thus the beneficial function of IL-1β as an immune regulator. Moreover, in chronic inflammatory state, sustained IL-1β is able to promote tumor development and progression[277]. Interestingly, despite the tumor promoting effects, IL-1β have been reported to be increased in a number of anti-tumor therapeutics[282]. Therefore, between this wide range of cytokines that belongs to the IL-1 family, this master thesis focuses on the role of IL-1β in tumor immune evasion and cancer development. However, before analyzing the role of IL-1β in the development of cancer that has been reported through the years, let us briefly discuss the pathways of synthesis, processing and releasing of IL-1β.

1.3.2 Synthesis, processing and release of IL-1β

IL-1β is mainly expressed in myeloid cells[209, 210, 283]. The regulation, the processing of IL-1β from its inactive pro-form and the bioavailability of the mature bioactive IL- 1β cytokine is a complex and controlled process, and several mechanisms have been proposed. The key feature of this pathway, is that IL- 1β translated as a pro-peptide without leader sequences and proteolytic cleavage is required for its biological activity.

Synthesis. The first step in the synthesis of mature IL-1β is termed "signal 1 or priming signal" and implicates the initial activation of monocytes or macrophages by Toll-like receptor agonists, IL-1β itself, immune complexes, adjuvants, molecular motifs carried by pathogens called pathogen-associated molecular patterns (PAMPs) or endogenous molecules released from dead cells called danger-associated molecular patterns (DAMPs)[284, 285]. Once the cell has been activated due the binding of the initiating stimulus to cell surface IL-1 receptor (IL-1R) or Toll-like receptors (TLRs), the Toll/IL-1 receptor (TIR) domains of these receptors recruit the myeloid differentiation primary response gene 88 (Myd88)[286, 287] and its oligomerization initiates a signaling cascade of four phosphorylated kinases called interleukin-1 receptor-associated kinases (IRAKs)[288, 289, 290]. These four phosphorylation events are followed by the recruitment and the oligomerization of tumor-necrosis factor-associated factor 6 (TRAF6)[291], which results in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation through the phosphorylation of IκB kinase β (IKKβ) and IκB[289, 291] and finally in the transcription IL-1β. Then, IL-1β mRNA is translated into a 31 kDa pro-peptide (pro-IL-1β), which resides and accumulates in the cytosol of the immune cells.

Processing. The second step required for the transformation of pro-IL-1β into the mature form, is a second signal termed "signal 2 or activation signal" and includes the maturation cleavage of pro-IL-1β by the pro-inflammatory protease caspase-1[292]. This maturation process is dependent on activation of caspase-1. The activation of caspase-1 occurs via its recruitment to multiprotein platforms called inflammasomes. The assembly of several inflammasome complexes that include members of the NOD-like receptor (NLR) family of proteins, has been reported for the activation of caspase-1[293]. However, the inflammasome complex that is formed from the NLR family member NLRP3 is one of the most studied inflammasomes and is accused of the activation of caspase-1. Thus, the inflammasome complex recruits and activate the caspase-1 enzyme that converts the 31 kDa pro-peptide pro-IL-1β to a 17 kDa mature IL-1β secreted cytokine[148, 294]. The cleavage site of caspase-1 resides in alanine at position 117 (D117), nine amino acids downstream the consensus sequence AXD of pro-IL-1β[292, 295].
Contrary to the above, the caspase-1-dependent processing of IL-1β is not the only mechanism for its maturation, as many studies have reported serine proteases (proteinase 3, elastase and cathepsin-G) derived from neutrophils and macrophages, which substitute the enzymatic activity of caspase-1 and cleave the IL-1β pro-peptide into the 17 kDa mature form[296, 297, 298]. Moreover, chymase and chymotrypsin are another two serine proteinases that have been identified to take part in the processing of pro-IL-1β. Last, metalloproteinases such as Meprin and proteases derived from invading pathogens, are proteinases that also mature pro-IL-1β[299, 300, 301, 302].

Secretion. As already mentioned, IL-1β is generated in the cytosol of the cells and its maturation cleavage has been reported to be essential for its secretion via an unconventional secretory pathway, which is independent from the conventional endoplasmatic reticulum (ER)/Golgi trafficking route. This conventional protein secretion pathway, which includes the ER together with the Golgi apparatus, allows the translocation of a vast majority of proteins through these cellular compartments and thus forming an endo-membrane system, before reaching their extracellular destination[303]. The main feature that characterize this pathway is a signal sequence at the N-terminus of the nascent peptide, the signal recognition particle (SRP), which is recognized by the SRP receptor (SR) located on the ER membrane and thus the nascent peptide starts its journey through this endo-membrane system receiving post-translational modifications before its final destination[304, 305]. However, IL-1β is a protein that takes an

unconventional secretory pathway to exit the cell and the reason is the lack of the signal peptide[306], a knowledge that led Rubartelli et al. in the completely blockage of conventional pathway of activated monocytes with brefeldin A, a fungal metabolite that inhibit the protein transport through the collapse of Golgi apparatus and intervention with its function leading to the accumulation of most cytokines at the ER/Golgi trafficking route, reporting that this kind of blockage did not affect IL-1β cytokine secretion, except only the secretion of cytokines such as IL-6 and TNFa, thus indicating IL-1β secretion is independent of conventional secretion pathway and follows a non-conventional secretion route[307].

As it is cleared by now, when we discuss about IL-1β secretion is essential to take into consideration that the mechanisms of release may be influenced by the strength and the type of stimulation, where most of the times is accompanied by activation of inflammasome complex, as described on (**Table 3**). Thus, the secretion of IL-1β can follow several different routes. The first route, relates to the increase of intracellular calcium that leads to the release of mature IL-1β through specialized secretory lysosomes[308, 309, 310, 311]. Another pathway by which IL-1β can be secreted is via microvesicular body-derived exosomes[312]. This exosome-dependent IL-1β is similar to secretory lysosome route, including the need for calcium influx and the content of the vesicle such as NLRP3, pro-IL-1β and caspase-1 for the maturation process. The unique feature of these secretory vesicles is that they also contain major histocompatibility complex (MHC) class II molecules, which are also secreted in the extracellular compartment together with mature IL-1β. In pyroptotic cells and especially in macrophages cell death, IL-1β secretion is often correlated with cell lysis via the NLRP3-dependent gasdermin N channel[313, 314, 315]. This secretory mechanism is based on caspase-1 cleavage of both gasdermin D precursor to produce gasdermin N and IL-1β pro-form. Gasdermin N then polymerizes and a pyroptotic pore is formed on plasma membrane for the release of mature IL-1β. This process can be considered as a third secretory pathway of mature IL-1β, in which the IL-1β and gasdermin N channel formation are dependent on caspase-1 and results in pyroptotic cell death[316, 317, 318]. However, in human blood monocytes the secretion of mature IL-1β can be cell death independent[276, 311, 314]. So, depending on monocyte's activation state, the IL-1β can be released via the gasdermin N channel, as already mentioned, or via a fourth secretory route, which is plasma membrane microvesicle-mediated[311]. The fifth and last caspase-1 independent mechanism that leads to IL-1β localization in the extracellular compartment, is under cellular stress conditions (hypoxia, necrosis or misfolded proteins), where the plasma membrane loses its integrity and this loss is accompanied by the release of many intracellular components, between them the lactate dehydrogenase (LDH) and the pro-IL-1β. The maturation process is mediated by extracellular proteases that cleave IL-1β pro-form near the cleavage site of caspase-1, resulting in mature IL-1β cytokine[319, 320].

1.4 The role of IL-1β-NLRP3 inflammasome axis in cancer

Through the years, cancer has been viewed as a process that originates from by cell-autonomous defects and its progression depends on the stimulatory or inhibitory signals that takes from its microenvironment. Cancer development and progression can be the result of a chronic inflammation, under which cancer cells have the ability to escape from or suppress the immunosurveillance mechanisms. Therefore, depending on environmental signals that either promote inflammation or induce immune responses, the carcinogenesis can be stimulated or suspended, respectively. Inflammasomes and their effectors, such as IL-1β, are able to shape the tumor milieu through their contribution to inflammation and immune responses, affecting thus the development, progression and treatment of cancer, which depicts the diverse roles of inflammasomes in modulating carcinogenesis and their potential targeting in translational research.

1.4.1 Tumor promoting effects of IL-1β-NLRP3 inflammasome axis

Despite the physiological role of inflammasome activation in maintaining homeostasis and defend the host from pathogenic insults, persistent activation of inflammasome results in chronic inflammation, which in turn contributes to tumorigenesis[143]. Result of this continuous activation is the release of the inflammasome effector cytokines IL-1β and IL-18 during this chronic inflammation state. IL-1 has been reported to play a critical role in carcinogenesis and tumor development[321], due to its function as downstream mediator of inflammation. Supporting evidences documented upregulation of IL-1β across many cancer types, including fibrosarcoma, lung cancer, pancreatic cancer, melanoma, breast cancer and colorectal cancer[322, 323, 324, 325]. In addition, alteration of NLRP3 expression and activity combined with high levels of IL-1β in plasma and tissues, are bad prognostic biomarkers in experimental tumor models and in cancer patients and associated with carcinogenesis and tumor invasiveness[189, 326, 327, 328]. The pro-tumorigenic effects of the IL-1β-NLRP3 inflammasome axis are associated with the ability of IL-1β to act as a

mediator of chronic non-resolving inflammation[321], tumor angiogenesis, endothelial cell activation and recruitment of immunosuppressive cells and thus resulting in immunosuppression, tumor development and progression, and metastasis[321].

Immunosuppression. The main characteristic of tumor microenvironment (TME) is the dominance of immunosuppression. The immune system as a response to the invading tumor cells launches the recruitment of inflammatory cells in TME as an antitumor response[329]. Despite the influx of inflammatory cells, cancer cells, through a wide range of mechanisms, manage to escape the immune surveillance, mainly through secretory factors, such as inflammasome-dependent IL-1β cytokine, and co-stimulatory surface molecules, from both the tumor cells themselves and the cells exist in TME, thus shaping the appropriate immunosuppressive conditions for tumor development[330].

Myeloid-derived suppressor cells (MDSCs) are key components of TME and considered to be main contributors of its immunosuppressive nature[331]. The tumor promoting role of IL-1β it was revealed when xenograft tumors which overexpressed IL-1β showed significantly greater accumulation of MDSCs and also a more rapid tumor growth compared to control mice transplanted with the same tumors but not secreting IL-1β[332, 333]. The IL-1β-induced MDSCs also downregulated the tumor immunity by inhibiting the activation of $CD4^+$ and $CD8^+$ T lymphocytes[333]. Notably, resection of large tumors of IL-1β-secreting cells restored immune reactivity within 7-10 days[332]. Additionally, tumor-bearing mice treated with an IL-1 receptor antagonist (IL-1a) that blocks the IL-1R signaling[332, 334], IL-1 receptor (IL-1R)-deficient ($IL\text{-}1R^{-/-}$) tumor-bearing mice[335] and IL-1β-deficient ($IL\text{-}1\beta^{-/-}$) tumor-bearing mice[336] exhibited delayed accumulation of MDSCs and suppressed tumor growth.

Moreover, the direct effect of IL-1β in the induction of neoplasia is shown in gastric adenocarcinoma, where the overexpression of IL-1β was sufficient to induce spontaneous and stepwise development of inflammation, the early recruitment of MDSCs and their activation for secretion of pro-inflammatory cytokines and thus the development of gastric carcinoma. Again, blocking of IL-1R signaling suppressed MDSCs accumulation and inhibited the tumor development[330]. The translational significance of IL-1β is underlined in melanoma patients, in whom the serum levels of IL-1β were significantly elevated and this elevation were accompanied by an increased frequency of MDSCs and Tregs[337]. The progression of melanoma found to be associated with elevated concentrations of IL-1β as compared to patients with stable disease, and enrichment of circulating monocytic MDSCs significantly correlated with a decreased progression free survival of melanoma patients[337]. Despite the direct effects of IL-1β on MDSCs propagation, IL-1β promotes the production of CC-chemokine ligand 2 (CCL2) in macrophages and tumor cells, and thus increased levels of CCL2 in TME results in the recruitment of C-C chemokine receptor type 2 expressing ($CCR2^+$) myeloid cell types into tumor tissue, such as MDSCs and TAMs[334]. IL-1R blockade attenuates tumor growth, decreases the number of tumor infiltrated myeloid cells and improves survival rate, in murine mammary gland tumor model and human breast cancer xenograft model[334].

The immunosuppressive function of IL-1β as a master cytokine in tumor progression, is also evident in the synergistic role of its blockade with anti-programmed death-1 (anti-PD-1) immunotherapy for tumor abrogation. Blocking of IL-1β, resulted in mammary tumor regression, a decreased tumor infiltration with $CCR2^+$ myeloid cells and thus a reduced immunosuppression with a greater antitumor immunity and improved tumor killing[338]. When IL-1β inhibition used in combination with anti-PD-1 checkpoint inhibitor, an enhanced antitumor immunity free of the immunosuppression mediated by IL-1β and an abrogated tumor progression occurred, unlike the treatments with either anti-IL-1β or anti-PD-1 Abs that partially reduced tumorigenesis[338]. Additionally, it has been reported that IL-1β upregulates the expression of programmed death-ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase (IDO) in macrophages and DCs, which inhibit NK cell-mediated and cytotoxic T lymphocyte-mediated cytotoxicity, leading to immunosuppression[339]. IL-1β neutralization or caspase-1 inhibition reversed this induction of PD-L1 and IDO expression, while blockade of PD-L1 and IDO increased the cytotoxic activity of NK cells[339]. Together, all these studies illustrate the tumor promoting functions of the inflammasome's effector cytokine IL-1β.

The pro-tumorigenic function of IL-β-inflammasome axis can be further demonstrated by targeting components of the inflammasome, mainly the Nlrp3 and caspase-1 proteins, that regulate the effector cytokine IL-1β. Similar to IL-1β findings, has been documented the critical role of NLRP3 for the intratumoral accumulation of MDSCs and the suppression of anti-tumor immune responses. In melanoma-bearing mice was established for the first time that the Nlrp3 inflammasome component impaired the antitumor T-cell immunity after dendritic cell (DC) vaccination and Nlrp3 expression promoted migration of MDSCs into the tumor[176]. In contrast, limiting Nlrp3 signaling using Nlrp3-deficient ($Nlrp3^{-/-}$) mice, resulted in an improved survival and immune response of host mice after DC vaccination,

based on a greater $CD8^+$ T cell response and a reduction of tumor infiltrated MDSCs, due to the impaired migration of $NLRP3^{-/-}$ MDSCs in tumor site[176].

The tumor promoting effects of inflammasome was also demonstrated by the specific targeting of caspase-1 inflammasome component in human MDSCs. MDSCs from squamous cell carcinoma patients showed an increased caspase-1 activity corelated with increased IL-1β expression[175, 260]. *In vitro* and *in vitro* studies documented the ability of caspase-1 in MDSCs to promote in a T cell–independent way tumor proliferation, whereas the genetic and pharmacologic inhibition of caspase-1 in MDSCs blunted tumor growth rates and promoted an antitumor response[175]. Also, pharmacologic blockade of NLRP3 inflammasome activity using MCC950 in squamous cell carcinoma mouse model resulted in a remarkably reduction of IL-1β production accompanied by a delayed tumorigenesis, a reduced immunosuppressive cell accumulation, including MDSCs, regulatory T cells (Tregs) and tumor-associated macrophages (TAMs), and a promotion of an antitumor immune response by increasing the numbers of effector T cells[260]. Additional findings obtained from sarcoma and melanoma mouse models support that the deleterious effects of Nlrp3 expression was owing to the suppression of the natural killer (NK) cell-mediated antitumor response and thus promotion carcinogenesis[340]. Moreover, the expression of Nlrp3 in tumor-associated macrophages (TAMS) governs their expansion and promotes the immunosuppressive $CD4^+$ T cell polarization, while suppressing Th1 cell differentiation and cytotoxic $CD8^+$ T cell activation in the TME of pancreatic ductal adenocarcinoma (PDA) via IL-1β[162]. Inhibition of NLRP3 signaling is associated with the immunogenic reprogramming of TAMs and T cells in TME[162]. Similar findings were obtained from mouse and human breast carcinoma, where Cancer-Associated Fibroblasts (CAFs) were reported to orchestrate tumour-promoting inflammation. CAF-derived inflammasome signaling and subsequent IL-1β secretion facilitated tumor progression by modulating the immune cell milieu towards a tumor-tolerating and immune suppressive phenotype, including the increased recruitment and the immunosuppressive activity of MDSCs[170]. The specific ablation of inflammasome signaling, through the knock-out or knock-down of Nlrp3 or Il-1β genes, resulted in the attenuation of tumor growth and a reduced accumulation of MDSCs into the tumor microenvironment[170]. Additionally, another study using mouse and human breast cancer models demonstrated that tumor progression was correlated with NLRP3 inflammasome activation and elevated levels of IL-1β at tumor tissues, whereas a total ablation of inflammasome signaling attenuated mammary tumor growth and improved survival rate in $Nlrp3^{-/-}$ and $Casp1^{-/-}$ mice[334]. Furthermore, the levels of IL-1β in tumor tissues together with the numbers of tumor infiltrated TAMs and MDSCs, were significantly decreased in inflammasome deficient mice, compared to that of control mice[334]. The tumor promoting effects of IL-1β-NLRP3 inflammasome axis have been also documented by the induction of a cancer-promoting cytokine, the IL-22, which is associated to a more aggressive phenotype of various cancer types, including breast, lung, gastric and skin cancers. The NLRP3-dependent IL-1β secretion from both myeloid and T cells is critical for IL-22 production and secretion by the $CD4^+$ T cells, resulting in cancer progression[180]. Blocking of NLRP3 inflammasome and IL-1β signaling diminished IL-22 production and subsequently reduced tumor burden[180].

Furthermore, the NLRP3 inflammasome blunted the effectiveness of the two clinically used anticancer chemotherapeutic agents gemcitabine and 5-fluorouracil, promoting thus tumor growth. These chemotherapeutic drugs activated the NLRP3 inflammasome in MDSCs, which in turn released IL-1β. MDSC-derived IL-1β induced the secretion of IL-17 by $CD4^+$ T cells, limiting anticancer immunity[43]. Accordingly, these two agents exerted higher antitumor affects when administered in $Nlrp3^{-/-}$ or $Casp1^{-/-}$ mice or wild-type mice treated with interleukin-1 receptor antagonist (IL-1Ra) that blocked IL-1R signaling, suggesting that chemotherapy-mediated activation of NLRP3 in MDSCs limits its antitumor efficacy and is a positive regulator of cancer growth[43].

NLRP3 inflammasome not only affects the effectiveness of chemotherapy, but also affects the immune escape mechanisms in immunotherapeutic strategies. A study this year identified a mechanism in which NLRP3 inflammasome signaling pathway is implicated in the resistance to anti-PD-1 immunotherapy in advanced melanoma patients and a melanoma mouse model[341]. In response to PD-1 blockade, CD8+ T cells were activated and triggered a PD-L1-NLRP3 inflammasome signaling cascade in cancer cells, leading to the intratumoral accumulation of MDSCs and thus the dampening of anti-tumor immune responses[341]. The genetic and pharmacologic inhibition of NLRP3 inflammasome, resulted in a suppressed recruitment of MDSCs, enhanced the anti-tumor immune response and suppressed tumor progression, enhancing thus the efficacy of anti-PD-1 antibody immunotherapy[341].

Overall, all these together make clear that NLRP3 inflammasome and its effector cytokine IL-1β promote immunosuppression by the accumulation of immunosuppressive cell populations, such as MDSCs, the induction of immune checkpoint molecules expression, the induction of tumor-promoting cytokine secretion and thus the

impairment of antitumor immune responses, demonstrating a crucial function of IL-1β-NLRP3 inflammasome axis in tumorigenesis.

Angiogenesis. The inflammasome effector cytokine IL-1β promotes tumor development not only by the shaping of the appropriate immunosuppressive tumor microenvironment, but also by influencing angiogenesis, a process by which new capillaries and vessels emerge, emerging from pre-existing vasculature, are sprouted for the needs of rapid tumor growth, development and invasion[336, 342, 343]. Microenvironmental IL-1β has been accused to cause *in vivo* angiogenesis and invasiveness in multiple tumor types[343]. Confirming this ascertainment, mice implanted with IL-1β secreting tumors showed enhanced tumor hyperneovascularization as evidenced by the elevated secretion of proangiogenic factors, such as vascular endothelial growth factor (VEGF), CXCL2 chemokine and hepatocyte growth factor, by malignant cells into the tumor microenvironment and by the increased density of blood vessels in tumors[32, 344, 345, 346]. Additional findings supporting the proangiogenic role of IL-1β, as in a model of melanoma myeloid cells reported to produce IL-1β, which in turn triggered endothelial cells to secrete VEGF and other proangiogenic factors, promoting thus the angiogenesis and tumor progression[347]. Furthermore, *in vivo* studies demonstrated that blocking of IL-1β secretion, either using genetic inhibition like IL-1β-deficient mice or pharmacologic inhibition such as recombinant IL-1Ra and antibody-mediated IL-1β neutralization, all resulted in the inhibition of growth of blood vessel networks in different cancer models, including B16 melanoma and mammary adenocarcinoma models[343, 348]. Additionally, these sites with the reduced vascularization also contained significantly decreased levels of VEGF[348]. Other studies showed that IL-1β-mediated hypoxia-inducible factor 1α (HIF-1α) upregulation triggered the induction of proangiogenic factor VEGF, further supporting the proangiogenic role of IL-1β[349]. All these studies reveal a cross talk between VEGF and IL-1β that takes place during tumor development, as are both needed for the tumor vascularization and the one seems to induce the other. Moreover, except from the induction of VEGF, IL-1β stimulated the production of CCL2 chemokine in macrophages and tumor cells[334], which has been reported to have multiple pro-tumorigenic functions, between them the angiogenesis and tumor progression[350].

Metastasis. IL-1β has also been shown, in a variety of cancer types, to pave the way for the tumor cell metastasis as well. Tumor metastasis is a process with high complexity and involves sequential events, including the dissemination of tumor cells from their primary foci, their infiltration into the blood circulatory system and their transition through it, and finally their capture in capillaries which resulted in the formation of metastatic nodules in distant target organs[351]. The occurrence of tumor metastasis in the target organ, requires the construction of an appropriate microenvironment, prior to the arrival of tumor cells, that favors the development of tumor and here comes the role of IL-1β in metastatic process[352]. IL-1β facilitate the invasion of cancer cells into the vascular system and triggers the expression of adhesion molecules, which allow the dissemination and implantation of tumor cells into the target tissue, on endothelial and cancer cells[353]. *In vivo* studies confirming this finding demonstrated that the numbers of MDSCs was significantly elevated in the lungs of melanoma-bearing mice prior to the arrival of cancer cells and the MDSCs-depended IL-1β secretion resulted in the expression of the adhesion molecule E-selectin in endothelia cells in the premetastatic niche, which is associated with increased metastasis in the lung tissue[354]. At the primary tumor site, malignant cells have the ability to secrete different growth factors enabling thus the distant engraftment and resulting in a chemokine and cytokine induction in the metastatic tissue. Therefore, both in primary and metastatic tumor tissue increased levels of IL-1β are detected[334]. Moreover, IL-1β levels have been documented to be associated with the implantation and liver metastatic growth in a melanoma model[6, 355]. Intravenous administration of IL-1β resulted in an enhanced hepatic metastasis of intrasplenically injected B16 melanoma cells[6]. This was consistent with previous findings showing that injection of mice with human recombinant IL-1β prior to the administration of melanoma cells induced an augmentation of lung metastases[356]. Supporting evidence for the key role of IL-1β in metastasis emerged from *in vivo* studies, in which hepatic metastasis of intrasplenically injected B16 cells was significantly reduced in IL-1β-deficient mice[353]. Ablation of IL-1β secretion using melanoma-bearing IL-1β$^{-/-}$ mice, reduced lung metastasis and improved their survival rate[343]. Similar findings were obtained from the systemic administration of IL-1Ra, which minimized the size and the numbers of hepatic metastases and also increased survival of a melanoma mouse model[355]. Interestingly, IL-1β-stimulated B16 melanoma metastasis seems to be tissue-specific as the administration of IL-1Ra except from the reduction of hepatic metastases also reduced B16 metastasis in bone marrow, spleen, lung, pancreas, skeletal muscle, adrenal gland and heart, whereas failed to reduce metastases to the kidney, testis, brain, skin and the gastrointestinal tract[357]. Additionally, a key step in the metastatic process is the loss of epithelial markers and the acquisition of

mesenchymal traits by the tumor cells[358]. IL-1β has been suggested to induce this mesenchymal fate through a process that is called epithelial-to-mesenchymal transition (EMT)[359]. Two of the hallmarks of EMT are the downregulation of E-cadherin expression, which is a suppressor of metastasis during tumorigenesis[11], and the induction of SNAIL expression, which is a prominent inducer of EMT[360]. Both of these proteins have been shown to be regulated by IL-1β in squamous cell carcinoma[361, 362] and gastric cancers[363]. Additionally, in breast cancer cells treatment with anti-IL-1β antibody attenuated the EMT phenotype[364]. Tumor invasion also requires matrix metalloproteinases (MMPs) for the degradation of the extracellular matrix[365] and one of them, the MPP9, has been reported to be upregulated by IL-1β, by the induction of transcriptional activity of the activator protein 1 (AP-1)[366]. An IL-1β dependent upregulation of vascular cell adhesion molecule-1 (VCAM-1) is also occurred in hepatic sinusoidal endothelium, promoting thus the metastatic process by increasing the adhesion of melanoma cancer cells to hepatic sinusoidal endothelium[353, 367].
Similarly, the key role of inflammasome activation in metastatic process is not only obvious by the effects of its effector cytokine, IL-1β, but also from studies targeting NLRP3 inflammasome components. In both murine and human breast cancer models, tumorigenesis was associated not only with elevated levels of IL-1β in primary and metastatic sites but also with induced activation of inflammasome components at both sites. However, a study reported that hepatic metastasis induced by intrasplenic injection of colon cancer cells was developed in $Casp1^{-/-}Casp11^{-/-}$ mice and $Nlrp3^{-/-}$ mice but in an IL-1β independent way[368]. In contrast, a similar and more recent study showed the key role of NLRP3-dependent IL-1β secretion in metastasis, as its secretion from macrophages induce a more migratory phenotype of tumor cells *in vivo*, resulting thus colon cancer cells to metastasize to the liver in mice[163]. Similar findings were obtained with the $Casp1^{-/-}$ and $Nlrp3^{-/-}$ mice exhibiting a significantly reduced lung metastasis after the orthotopic implantation or intravenous administration of breast cancer cells, confirming thus the hypothesis that the NLRP3 inflammasome activation contributes to the metastatic process[334]. Specifically, a study reported that the NLRP3 expression, activation and subsequent IL-1β secretion in tumor-associated macrophages (TAMs) was associated with tumor lymphangiogenesis and the induction of pulmonary metastasis via the lymphatics in breast cancer mouse model[166]. These findings were also confirmed in mammary carcinoma patients, where the NLRP3 expression in tumor-infiltrating macrophages correlated with survival, lymph node invasion and metastasis[166]. In melanoma or prostate carcinoma metastasis models, is suggested that NLRP3 induces metastasis but in an independent of inflammasome activation way, as $Nlrp3^{-/-}$ mice rather than $Casp1^{-/-}$ mice had decreased numbers of lung metastases after the inoculation of cancer cells and this response was attributed to the increased NK cell activity in $Nlrp3^{-/-}$ mice, compared to the mice that expressed the NLRP3[340]. Additional findings from two different studies supporting that despite the similar pulmonary metastasis coverage between the $Casp1^{-/-}Casp11^{-/-}$ and wild-type mice in a MMTV-PyMT model of breast cancer[369], the implantation of tumors derived from MMTV-PyMT mouse models in $Nlrp3^{-/-}$ mice led to reduction of lung metastasis[334]. Like IL-1β, so can NLRP3 promote the EMT phenotype by inducing TGF-β1 signaling and SMAD activation, a signaling pathway that mediates EMT in a way that is independent of caspase-1 and inflammasome's effector cytokines[370, 371].

1.4.2 Anti-tumorigenic effects of IL-1β-NLRP3 inflammasome axis

Although the well-documented clinical and experimental evidence of pro-tumorigenic role of IL-1β-NLRP3 inflammasome axis, its role in cancer remains controversial. There is growing evidence that NLRP3 inflammasome activation and the subsequent secretion of inflammasome products, particularly IL-1β, have tumor-suppressive effects and a key role in the stimulation of adaptive immune responses and anticancer immunosurveillance[372, 373].
The tumor-suppressive effects of IL-1β have been early assessed and highlighted by the potential of recombinant IL-1 to induce anti-tumorigenic affects, which was correlated with the ability of IL-1 to induce type 1 and type 17 antigen-specific T cell responses. Several years ago, Nakamura and colleagues reported that intratumoral administration of IL-1α in different murine tumors, such as sarcoma, melanoma and colon adenocarcinoma, resulted in their regression[374]. Some years after, North and coworkers demonstrated with their study, for the first time, the anti-tumorigenic role of the inflammasome's effector cytokine IL-1β, where the intraperitoneal injection of recombinant human IL- 1β resulted in the regression of murine tumors, including SA1 sarcoma and L5178Y lymphomas, with the regression occurred after the third day of tumor progression and administration of IL- 1β, thus being the first indication that the action of IL- 1β is based on an underlying host-immune response, which is generated after the third day of tumor development[375]. A second evidence for the IL-1β-induced host immunity leading to tumor regression was provided by the inability of IL-1β to suppress tumor growth in T-cell-deficient mice, suggesting that IL-1β-induced tumor regression is depended by the induction of T-cell mediated anti-tumor responses[375]. Besides the therapeutic effects of IL-1β administration, Allen *et al.* demonstrated the protective role

of IL-1β in mouse models of chemically induced colitis and colitis-associated cancer, the attenuated levels of IL-1β at tumor site were associated with the increased tumor burden[376]. Moreover, elevated levels of IL-1β have been consistently associated with successful cancer immunosurveillance[377]. Confirming evidence that further developed this finding came from a recent study showing that *in vivo* administration of neutralizing antibodies for tumor necrosis factor α (TNFα), TNF-related weak inducer of apoptosis (TWEAK) or TNF-related apoptosis-inducing ligand (TRAIL) did not affect myeloma elimination by Th1 cells, which was, however, severely impaired by the *in vivo* administration of recombinant IL-1Ra that neutralized the receptor for both IL-1α and IL-1β[378]. Moreover, IL-1β has not only a key role for T-cell mediated immunity against cancer, but also is critical for the induction of tumoricidal activity in macrophages, as *in vivo* blockade IL-1α and IL-1β with IL-1Ra impaired the activation and tumoricidal activity of tumor-infiltrating macrophages[378].

As understood from the above, the administration of recombinant IL-1 exerted tumor-suppressive effects in murine tumor models. However, in several clinical trials the systemic administration of IL-1 to human cancer patients yielded modest anti-tumor effects against melanoma, renal cell carcinomas or other malignancies, and may not be worth the toxicity necessary to achieve them[379]. For this reason, IL-1 was encapsulated into microspheres, so to delivered locally and prevent the cytotoxic effects of IL-1 systemic administration[380]. These IL-1 loaded microspheres were avidly taken up by macrophages and activate them so to participate in anti-tumor immune responses[380, 381]. The intratumoral administration of these IL-1 microspheres into fibrosarcoma-bearing mice, resulted in optimal survival of mice, regression rates of tumor development, tumor cells necrosis and infiltration of leukocytes, confirming thus the immunotherapeutic efficiency of microspheric IL-1[380, 381].

The tumor-suppressive function of IL-1β-NLRP3 inflammasome axis, besides the *in vivo* administration or targeting of the inflammasome effector cytokine IL-1β, can be also demonstrated by targeting NLRP3 inflammasome components in the hematopoietic[376] and non-hematopoietic compartment[173, 382, 383], reflecting nevertheless the effects of IL-1β in the anti-tumor immune response. Tumor cell necrosis associated with the type 1 and type 17 immune responses and chemotherapy is sensed by the NLRP3 inflammasome via the release of mitochondrial ATP from dying cells, further supporting the IL-1β signaling cascade[384]. The dying tumor-derived ATP has been reported to trigger the NLRP3 inflammasome of DCs allowing the subsequent secretion of IL-1β[167]. IL-1β production by DCs appeared to be crucial for the therapeutic efficacy of anti-cancer chemotherapy, as their cross-presentation ability of neo-antigens from dying cancer cells resulting in the induction tumor-specific interferon-γ (IFN-γ)-producing T lymphocytes[167]. Mice deficient in inflammasome components (Nlrp3$^{-/-}$ and Casp1$^{-/-}$) and mice deficient in the IL-1 receptor-1 (Il1r1$^{-/-}$) failed to induce IFN-γ-producing CD8^{+} T cells by dying tumor cells and thus anticancer chemotherapy turned out to be inefficient against tumors established at each of these hosts[167]. However, injection of recombinant IL-1β fully restored deficient T cell priming in Nlrp3$^{-/-}$ or Casp1$^{-/-}$ mice[167]. Extending these findings, it has been also demonstrated that the CD8^{+} T cell- and IFN-γ-dependent therapeutic outcome of chemotherapy treatment in established tumors requires both IL-1β and IL-17 cytokines, as mice deficient in IL-1β or IL-17 signaling abrogated the therapeutic effect[385]. Moreover, increasing evidence shows that inflammasomes also assemble in non-hemopoietic cells[383] and thus the anti-carcinogenic role of NLRP3 except from the hematopoietic compartment may also be demonstrated in non-hemopoietic compartment. In support, a study from Wei *et al.* found that the expression of NLRP3 inflammasome components in hepatic parenchymal cells was essential for preventing hepatocellular carcinoma[382]. Both mRNA and protein levels of NLRP3 were significantly decreased in hepatic parenchymal cells derived from liver cancer biopsies when compared with non-cancerous liver tissues, suggesting that NLRP3 inflammasome might suppress the development of human liver cancer[382]. In a model of liver metastasis, mice deficient in NLRP3 inflammasome components showed exacerbated colorectal cancer metastatic growth in the liver, whereas the activation of NRLP3 impacted maturation of hepatic NK cells, leading to an effective NK-cell-mediated tumor attack and suppressing thus liver colorectal cancer metastatic growth[368].

Additionally, tumor-inhibiting function has also been ascribed to IL-1β-NLRP3 inflammasome axis in case of colorectal cancer (CRC). The genetic absence of NLRP3 inflammasome components, such as Nlrp3, ASC and caspase-1 proteins, increased the susceptibility and morbidity to cancer and the number of colon polyps in colitis-associated cancer mouse models induced by the DNA-damaging agent azoxymethane (AOM) and chemical colitogen dextran sulfate sodium (DSS)[173, 174, 376, 386, 387]. Also, the increased tumor growth was associated with attenuated levels of intratumoral IL-1β[376]. However, one study suggested that mice deficient for NLRP3 (Nlrp3$^{-/-}$) developed a less severe DSS-induced colitis compared to wild-type, which was accompanied by lower levels of IL-1β in colonic tissue[388], whereas another study demonstrated that AOM-DSS-induced colonic cancer was enhanced in caspase-1-deficient (Casp1$^{-/-}$) mice but no differences in tumor prevalence were observed between NLRP3$^{-/-}$ and wild-type mice[389]. Also, colorectal tumorigenesis induced by AOM in the presence of a high-cholesterol diet was suppressed by the deletion

of NLRP3, suggesting that the cancer promoting effects are mediated by NLRP3 inflammasome activation[390]. These contrasting effects of IL-1β-NLRP3 inflammasome axis in colon tumorigenesis may be explained to a certain extent by the differences in the gut microbiota of experimental mice between the different animal facilities and the use of littermate controls. Confirming evidence came from a recent study, in which mice carrying a gain-of-function mutation $Nlrp3^{R258W}$ reported an enhanced IL-1β production in their colon tissue and were strongly resistant in the development of DSS-induced experimental colitis and CRC[391]. The induced levels of IL-1β contributed to the remodeling of intestinal microbiota by boosting local antimicrobial peptides, resulting in the local induction of regulatory T cells (Tregs) and thus to the maintenance of homeostasis and restriction of intestinal inflammation[391]. Furthermore, elevated levels of IL-1β production by myeloid cells lacking the protein tyrosine phosphatase non-receptor type 2 (PTPN2) protected from colitis-associated tumor development, whereas Inhibition of IL-1β increased tumor burden[392]. In this murine model, myeloid cell-specific deletion of PTPN2 resulted in induced inflammasome activation and subsequent increased production of IL-1β, as a consequence of the increased phosphorylation of the inflammasome adaptor molecule ASC, suggesting the inflammasome-mediated protection of IL-1β against CRC[392]. In contrast, a previous study had reported that $IL\text{-}1R^{-/-}$ mice were not more susceptible to AOM-DSS-induced colonic cancer than the wild-type mice[393], which opposes the beneficial effects of IL-1β in CRC. The answer was given by a recent study, which examined the impact of cell-type specific IL-1 signaling within CRC microenvironment[394]. Analysis of epithelial cell and T-cell specific IL-1R ablation revealed an alleviation of tumorigenesis in a murine model of CRC, demonstrating thus the pro-tumorigenic role of IL-1 in these cell types[394]. This pro-tumorigenic role in epithelial and T-cells was counteracted by its role in myeloid cells, particularly neutrophil populations, which was shown to be potently anti-tumorigenic due to their role in controlling tumor microbial infiltration and dampening CRC development, whereas myeloid cell specific IL-1R ablation caused bacterial invasion into tumors, heightened inflammation and aggressive CRC progression[394]. Except from colonic tumorigenesis, a similar approach was also reported in skin carcinogenesis, where the expression of ASC protein and the subsequent NLRP3 inflammasome activation was considered as a driver of tumorigenesis when expressed in infiltrating myeloid cells, whereas ASC expression in keratinocytes was identified as a tumor-suppressor, proposing thus the opposing functions for the ASC inflammasome component between tumor cells and infiltrating immune cells[395].

Overall, all these together make clear that IL-1β-NLRP3 inflammasome axis except from the pro-tumorigenic role that have been demonstrated in a number of studies, also exerts anti-tumorigenic effects with the most of them to being demonstrated in colon cancer. However, the protective or pro-tumorigenic activity of NLRP3 inflammasome signaling is strictly correlated to the cell lineage involved, a fact that determines the propensity for tumorigenesis.

1.5 Rational and Objectives

Remarkable advancements in recent years have greatly increased our knowledge in the NLRP3 function, as its activation has been accused not only for fighting bacterial, fungal and viral intruders but also for mediating cancer initiation and progression. Inflammasomes are now considered central signaling hubs of the immune system and the study of their activation and regulation is a rich field in immunology. Understanding the way that inflammasome signaling affects tumor development and progression has been one of the most intriguing areas in the field. The inflammasome signaling is closely associated with many human cancers and exhibits conflicting roles in multiple aspects of tumorigenesis. The effect of inflammasome's activation in tumor initiation and promotion can range from the suppression of tumor immunosurveillance, through the maintenance of an inflammatory and immunosuppressive microenvironment, to the fomentation of angiogenesis and metastasis. In contrast to its pro-carcinogenic activity, the anti-tumor function of inflammasome signaling pathway is largely reflected in its preventive function in colitis and colon cancer by the fostering of anti-tumor immunity and the maintenance of epithelial integrity, which have been reported in a number of studies. In addition, clinically used chemotherapeutic agents induce direct activation of the NLRP3 inflammasome, which supports tumor growth and development, unraveling thus an inflammasome mediated-mechanism of resistance to chemotherapy[43]. Furthermore, checkpoint blockade immunotherapy, which has revolutionized cancer therapeutics has been also associated with the induction of adaptive resistance and low response rates in a sizable portion of cancer patients. This impairment in success of immunotherapy may be is attributed to the immunosuppressive network of the tumor microenvironment, which in turn impede the elicitation of potent anti-tumor immune responses. However, little is known about the contribution of NLRP3 inflammasome to the immunosuppressive nature of tumor

microenvironment and thus to tumorigenesis and its role in shaping tumor responses to immunotherapy remains elusive[396]. These findings greatly highlight NLRP3 inflammasome as a novel target to prevent and treat cancer.

Although refinement of our understanding of NLRP3 activation during carcinogenesis continues, targeting of NLRP3 inflammasome as a therapeutic is still to be elucidated. Regarding the fact that NLRP3 inflammasome signaling is diverse across different tumor types, the understanding of how NLRP3 inflammasome activation and the subsequent IL-1β secretion affects tumor development and progression, will gain insight into how to manage this diversity, which is an important area for future investigations. Moreover, the tumor microenvironment is composed of by a heterogeneous mixture of cell types that either promote or suppress tumorigenesis and the relative expression of inflammasome components differs in these cell type variety[397], suggesting thus that inflammasomes perform distinct functions in different cell types and the specific targeting of NLRP3 inflammasome in particular cell types may be has beneficial effects. In addition, several studies have also reported that TLR-mediated activation of NF-κB in MDSCs by various proinflammatory factors, such as TLR ligands, IL-1β or TNF-α is able to contribute to the suppressive activities of MDSCs and correlate the IL-1β cytokine with recruitment and activation of MDSCs[57]. However, so far studies have focused on the role of NLRP3 inflammasome in the cell recruitment at tumor site, but which of the tumor-infiltrating cell subtypes the NLRP3 inflammasome activation has a key role in tumor development and progression has not been thoroughly investigated. Furthermore, clinical trials that use agents which specifically target NLRP3 inflammasome components in human cancers, are yet to be performed. Current treatment modalities that have been used to clinical trials are related with the targeting of the downstream effector molecule IL-1β, with the most of them be relatively non-specific and have low efficacy; therefore, there is an increasing need for the translational research on tumorigenesis the advancing to clinical trials new therapeutics that are specific for NLRP3 activation without compromising beneficial effects from other inflammasomes. Undoubtedly, future research on cancer care should include a combination of therapies, which will combine immunotherapy with therapies that targets the effector function of cell subtypes with a key role in TME.

Taking the above into consideration, for the present Master Thesis we formed the hypothesis that the increase of NLRP3 inflammasome activation is associated with tumor development and progression. In this project, we aim to investigate how the NLRP3 inflammasome and consequently IL-1β shapes tumor growth, anti-tumor immune response and immunotherapy responses.

To dissect our hypothesis, we set out to work with induced melanoma models and investigate the role of inflammasome during tumor development and in response to immunotherapy, performing experiments on transgenic mice that are deficient for NLRP3 inflammasome. Specifically, our first aim is to assess the effect of NLRP3 inflammasome and its effector cytokine IL-1β in tumor development and progression. Furthermore, among our primary objectives is to determine which of the cell subtypes that infiltrate the heterogeneous tumor microenvironment shows an increase expression in inflammasome components and an induced inflammasome activation and afterwards to determine the their inflammasome's functional role in tumor growth. Additionally, we are also plan to examine the effect of a combination therapy, which will combine the immunotherapy with the specific inhibition of the NLRP3 inflammasome, in tumor growth and tumor immune response. Ultimately, our goal is to focus our research on the deeper understanding of how the NLRP3 inflammasome activation in the context of melanoma contributes to tumor progression, and these findings can be translated for therapeutic purpose in clinical practice.

It is expected that the findings of this Master Thesis will shed light on the involvement of the NLRP3 inflammasome of specific tumor-infiltrating cell subtypes in the establishment of cancer and will open exciting new avenues for translational research with the potential for therapeutic targeting of tumorigenesis.

2. Materials and Methods

Animals. C57BL/6J mice were purchased from the Jackson Laboratory; NLRP3$^{-/-}$ mice (on a C57BL/6J background) were obtained from Institute of Molecular Biology and Biotechnology Institute [IMBB] and previously described by Tschopp et al.[398]. All mice were maintained in the animal facility of the Biomedical Research Foundation of the Academy of Athens [BRFAA] and Institute of Molecular Biology and Biotechnology Institute [IMBB]. Unless is indicated, in all experiments were used sex- and age-matched mice aged between 6 and 10 weeks.

PCR Genotyping. Screening of NALP3 deficient mice by PCR genotyping was carried out using the following primers on tail genomic DNA: 5'GCTCAGGACATACGTCTGGA (forward in intron 1), 5'TGAGGTCCACATCTTCAAGG (reverse in exon2) and 5'TTGTAGTTGCCGTCGTCCTT (reverse in EGFP cassette).

Cell lines. The cell line that was used for melanoma induction was the melanoma cell line B16.F10 and were kindly provided by A. Eliopoulos (Medical School, University of Crete, Heraklion, Greece).

Solid tumor induction. The transplantation of solid tumors in the tumor models was performed as described below. Mice were implanted subcutaneously, in the intradermal layer of the back of mice that were previously shaved and anaesthetized with isoflurane, with 3×10^5 B16.F10 melanoma cells (viability assessed by Trypan blue exclusion). Tumors became visible at day 10 and the tumor volume was monitored from day 10 to day 15 following melanoma cells inoculation. The tumor growth was monitored by measurement of two perpendicular diameters of the tumor by caliper and was calculated using the equation $\frac{(\text{length} \times \text{width}^2)}{2}$. Mice were euthanized and analysis was performed 9-15 days after the melanoma cells injection. At the endpoint of each experiment, the weight of spleen and tumor tissue of tumor-bearing mice was determined.

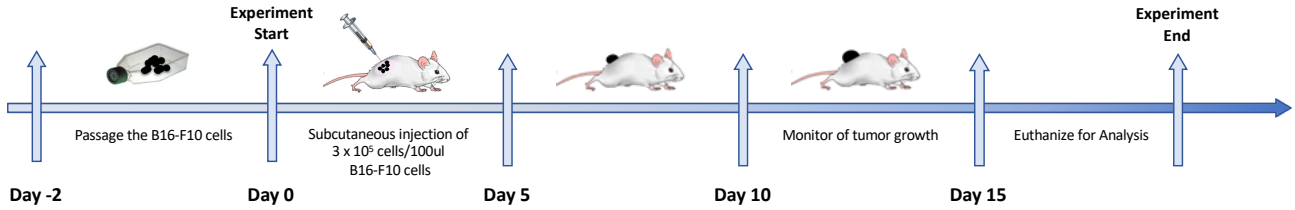

Figure 1. Protocol for melanoma induction in mouse models. Melanoma tumors were induced in 6-10 weeks old C57BL/6J (wild type) and NLRP3-/- mice injected with B16.F10 melanoma cells. Mice were euthanized and analysis was performed 9-15 days after the melanoma cells injection.

Cell isolation from tumors and lymphoid organs. For the analysis of Tumor-infiltrating lymphocytes (TILs), single-cell suspensions were generated by dissecting and dissociating tumor tissue in the presence of collagenase D (1 mg ml^{-1}, Roche) and DNase I (0.25 mg ml^{-1}, Sigma), diluted in RPMI medium (Gibco), for 45 minutes at 37°C and then were homogenized and strained passing through a 40-μm pore size cell strainer (BD Falcon). For the analysis of LNs and spleens, LN and splenocytes single-cell suspensions were prepared by homogenization and passing through a 40-μm pore size cell strainer.

Flow cytometry and cell sorting. Single-cell suspensions from TILs, spleen, or LNs were stained for extracellular markers incubated for 20 min at 4°C with the following anti-mouse antibodies: CD45-PerCP/Cy5.5 (BioLegend, clone 30-F11, #103132, diluted 1:200), CD11c-PE/Cy7 (BioLegend, clone N418, #117318, diluted 1:200), CD11b-Brialliant Violet 510 (BioLegend, clone M1/70, # 101263, diluted 1:200), Gr1-Pacific Blue (BioLegend, clone RB6-8C5, #108430, diluted 1:200), Ly6G-PE (BioLegend, clone 1A8, #127608, diluted 1:200), Ly6C-Brilliant Violet 421 (BioLegend, clone RB6-8C5, #108430, diluted 1:200), CD8a-PE/Cy7 (BioLegend, clone 53-6.7, #100722, diluted 1:200), CD4-PE (BioLegend, clone RM4-4, #116006, diluted 1:200) and NK-1.1-APC (BioLegend, clone PK136, #108710, diluted 1:200). Dead cells in cultured MDSCs/splenocytes were stained by addition of 7-AAD Viability Staining Solution (BioLegend, #420404). For NLRP3-APC (R&D Systems, clone 768319, #IC7578A, diluted 1:25) and IL-1β-FITC (R&D Systems, clone 166931, #IC4013F, diluted 1:50) intracellular staining, cells were stained for the extracellular markers, and then permeabilized and stained using the intracellular Fixation & Permeabilization buffer set (eBioscience) according to the manufacturer's instructions, whereas for Foxp3-Alexa 488 (BioLegend, clone 150D, #320012, diluted 1:50) intracellular staining, cells were fixed and stained using the Foxp3 Transcription Factor Staining Buffer Kit (molecular probes) according to the vendor's instructions. All samples were analyzed using ARIA

III (Becton Dickinson Biosciences). Flow cytometry data were analyzed with FlowJo v.8.7 software. $CD11c^{-}$ $CD11b^{+}Gr\text{-}1^{+}$ MDSCs were sorted on a FACS ARIA III (Becton Dickinson Biosciences). Cell purity was above 95%.

Cell culture. B16F.10 melanoma cancer cells were cultured at 37°C under 5% CO2 in RPMI (Gibco) medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and 0,1% Mercaptoethanol. Cells were passed when they were 90%-100% confluent. All experiments were performed with low passage (p2-3) B16F.10 melanoma cells.
Splenocytes and sorted MDSCs were obtained as previously described. Mouse splenocytes and MDSCs cells were grown in RPMI (Gibco) culture medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and 0,1% Mercaptoethanol. The stimuli at the cultures were added where indicated, as mentioned below.

Inflammasome activation assays. Splenocytes as well as sorted splenic MDSCs from naïve and tumor bearing mice were isolated as previously described. We seeded 5×10^5 splenocytes or 1×10^6 MDSCs per well in 96-well flat bottom and 96-well U bottom plates, respectively and were stimulated for 24 hours with 1 μg/ml Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 (Sigma, L2880). The next day, 1 hour before the ending of the stimulation time, the culture medium was supplemented with 5 μM adenosine 5'-triphosphate disodium salt hydrate (ATP) inflammasome activator (Jena Bioscience, NU-1010-1G) for 1 hour. Supernatants were removed and analyzed using Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit (R&D Systems, MLB00C), according to manufacturer's instructions, and cultured cells were prepared for staining of extracellular and intracellular markers, as described above.

MCC950-mediated inflammasome inhibition assays. Sorted splenic MDSCs or total spenocytes from Day 15 tumor bearing mice were seeded 5×10^5 cells per well in 96-well flat bottom plates and stimulated overnight with 1 μg/ml Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 (Sigma, L2880). The following day, in the medium from the overnight culture was added water for injection (control) or MCC950 NLRP3 inhibitor (0,5-2 μM) (Sigma-Aldrich, 5381200001) for 1 hour. After incubation time, cells were stimulated with 5 μM adenosine 5'-triphosphate disodium salt hydrate (ATP) inflammasome activator (Jena Bioscience, NU-1010-1G) for 1 hour. Supernatants were removed and used for mouse IL-1β ELISA and cultured MDSCs were stained again for CD11b and Gr1 and also prepared for staining of extracellular and intracellular markers, as described above.

Enzyme-linked immunosorbent assay (ELISA). Tumor and spleen homogenates were generated in phosphate-buffered saline (PBS) that was supplemented with a cocktail of protease inhibitors (Roche) using a pestle inside an eppendorf. Fresh mice blood was collected and centrifuged for serum isolation. The conditioned media of cultured splenocytes and MDSCs from in vitro cultures were also collected. The homogenates and cell culture supernatants were centrifuged and together with the serum were assayed for mouse IL-1β using Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit (R&D Systems, MLB00C), according to manufacturer's instructions.

Preparation of tumor explant supernatants (TES). TES were prepared from C57BL/6J mice injected with B16.F10 melanoma cells. At day 15, non-ulcerated tumors were excised, minced into small pieces and then digested in the presence of collagenase D (1 mg ml^{-1}, Roche) and DNase I (0.25 mg ml^{-1}, Sigma), diluted in RPMI medium (Gibco), for 45 minutes at 37°C. For the generation of single-cell suspensions the digested tissue pieces were homogenized and strained passing through a 40-μm pore size cell strainer (BD Falcon). After a cell wash with PBS, tumor cells were resuspended in RPMI (Gibco) medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and 0,1% Mercaptoethanol, plated in 6-well plates at a density of 10^7 cells/ml and cultured overnight. The following day, cell free supernatants were collected and kept at −80°C. 20% v/v tumor explant supernatants from B16.F10 melanoma cells were used in all experiments.

Data analysis and statistics. Data are presented as mean ±S.D., as bar graphs represent the mean and standard deviation (SD) between biologically independent mouse samples or technical repeats, as indicated each time. For statistical analysis, all data were analyzed using Prism 8 (GraphPad Software, Inc., La Jolla, USA). Data were analyzed using the two-tailed, parametric, unpaired Student's t test or the two-tailed, nonparametric Mann–Whitney test, as appropriate after testing for normality of the values with the F test, with 95% confidence intervals. For multiple-group comparisons of the parametric data, the one-way ANOVA test was performed, while the comparisons between the nonparametric data were performed using the Kruskal–Wallis test, again depending on normality of the values tested with the F test.

3. Results

3.1 Activation of NLRP3 inflammasome in myeloid cell compartment during tumor development and progression

3.1.1 Myeloid signature during melanoma tumor development

As previously mentioned, tumor microenvironment (TME) is composed of heterogeneous immune and nonimmune cell populations including tumor cells. Solid tumors, like melanoma, are known to have a strong myeloid component in tumor development. Myeloid cells have been accused to be the key players of microenvironmental regulation of tumor growth and development, with a critical role in therapeutic responses in cancer treatment[399]. Based on these, myeloid signature during melanoma tumor development was first assessed in melanoma-bearing mice at different stages of tumor growth in tumor and spleen tissue of these mice. To address the frequencies of myeloid cells, and more specifically those of MDSC and DC cell subtypes, we performed Flow cytometric analysis, which revealed that tumor progression was accompanied by a small decrease in the numbers of $CD11c^+$ DCs in tumors of B16.F10 melanoma cell (B16.F10)-inoculated animals (Figure **1b**), whereas the frequencies of $CD11c^-CD11b^+Gr1^+$ MDSC cell population were not altered (Figure **1b**), regarding the fact that the numbers of tumor-infiltrating $CD45^+$ leukocytes were significantly decreased (Figure **1a**). Taking into consideration the periphery of these mice, upon melanoma establishment spleen infiltrated DCs were notably reduced at the 8th day after tumor inoculation but at the endpoint their numbers were the same as the naïve state (Figure **1c**), whereas MDSCs were significantly enriched in the spleen of tumor-bearing mice (Figure **1c**).

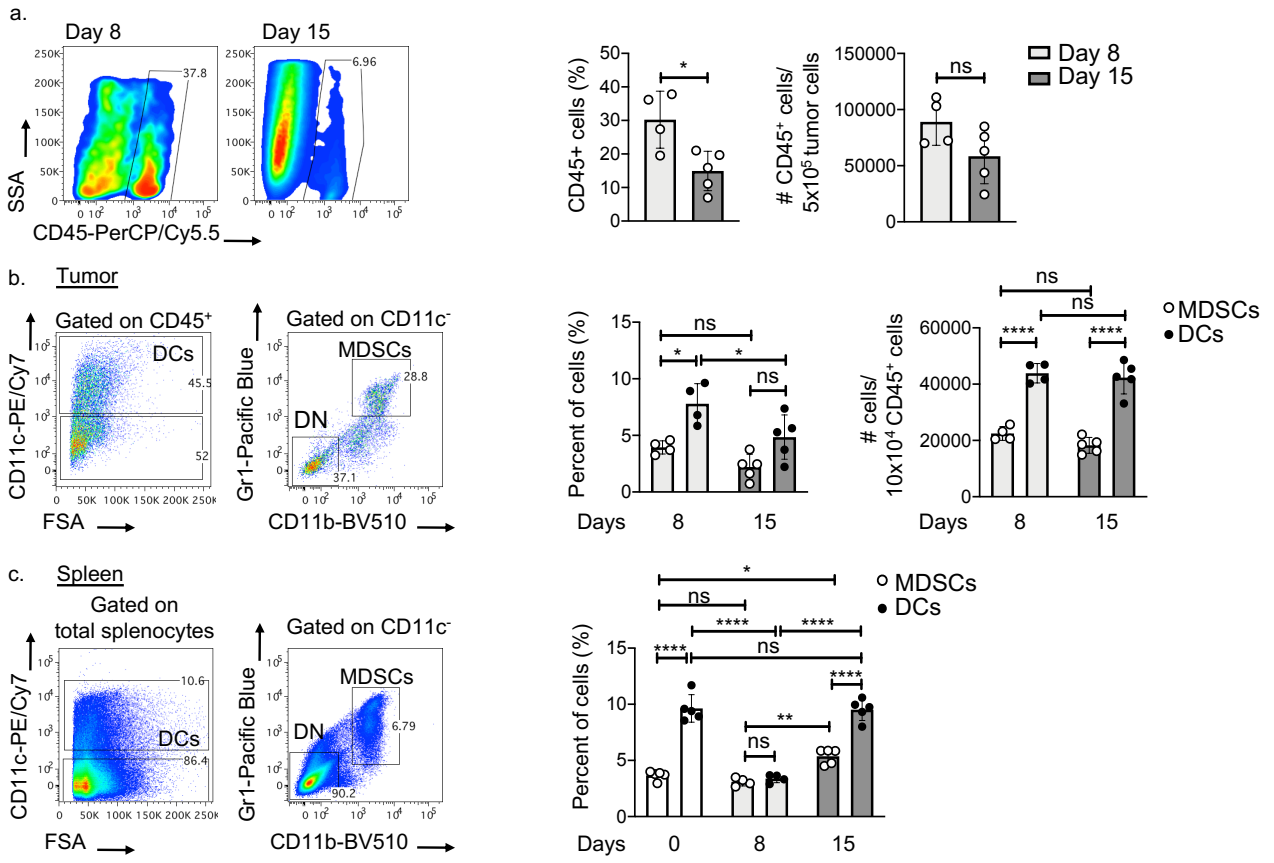

Figure 1. Increased myeloid cell infiltration in tumor tissue and peripheral lymphoid organs in an induced murine melanoma tumor model. (a) Representative FACS plots, frequencies of $CD45^+$ cells in total tumor cells and numbers per 5×10^5 tumor cells of $CD45^+$ cells. **(b)** Gating strategy, frequencies of intratumoral MDSCs ($CD11c^-CD11b^+Gr1^+$) and DCs ($CD11c^+$) in total tumor cells and their numbers per 10×10^4 $CD45^+$ cells. **(c)** Gating strategy and frequencies of spleen infliltrated MDSCs ($CD11c^-CD11b^+Gr1^+$) and DCs ($CD11c^+$) in total splenocytes. Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by unpaired Student's t test (**a**) or one-way ANOVA (**b** and **c**). Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

Because both DCs and MDSCs are heterogeneous populations, we also sought to determine how tumor development affects the frequencies of the respective DC and MDSC subsets. As already mentioned, a subset of DCs which may exhibit immunosuppressive properties are called inflammatory or monocyte-derived DCs and are characterized by the expression of Ly6C, CD11b, MHC-II and CD11c[109, 126]. To this end, we observed an increased recruitment of $CD11c^{+}CD11b^{+}Gr1^{+}$ ($Gr1^{+}$) cell subset at tumor site as the tumor developed, whereas in spleen the frequencies were not altered between the endpoint and the naïve state, but were also increased between the 8^{th} and 15^{th} day after melanoma induction (Figure **2a,b**). In addition, the $CD11c^{+}CD11b^{+}Gr1^{-}$ ($Gr1^{-}$) subset population was significantly decreased in spleen and tumor of melanoma mice as the tumor progressed (Figure **2a,b**). Regarding the MDSC subset populations, we observed a reduction in the numbers of intratumoral $CD11b^{+}Ly6C^{high}Ly6G^{-}$ monocytic MDSCs (M-MDSCs), whereas an increased recruitment of them was noted in the spleen (Figure **2a,b**). Despite the reduction of M-MDSCs at tumor site, intratumoral $CD11b^{+}Ly6C^{low}Ly6G^{+}$ polymorphonuclear (PMN-MDSCs) were notably elevated between the different days of tumor progression, whereas their frequencies in spleen tissue shown a slightly decrease (Figure **2a,b**).
Collectively, these findings demonstrate a gradual increase in the frequencies of $CD11b^{+}Gr1^{+}$ MDSCs in the spleens of tumor-inoculated mice compared with naïve animals, whereas frequencies of $CD11c^{+}$ DCs were not altered. Frequencies of tumor infiltrated MDSCs and DCs have no difference between the different stages of tumor development. Interestingly, both in spleen and tumor tissue the frequencies of DCs are notably elevated compared of those of MDSCs. Moreover, MDSCs in peripheral lymphoid organs, such as spleen, are largely represented by PMN-MDSCs ($Ly6G^{+}$), whereas in tumor the opposite seems to happens despite the gradual increase of PMN-MDSCs. DCs, on the other hand, are represented by $Gr1^{-}$ cell subset in spleen, in contrast to TME where the $Gr1^{+}$ are increased.

3.1.2 Increased IL-1β levels in TME and peripheral lymphoid organs during tumor development and progression

Based on the bibliography of the NLRP3 inflammasome, the activation of the NLRP3 inflammasome implies the secretion of the IL-1β cytokine. In order to ascertain the role of the inflammasome and consequently of its effector cytokine IL-1β during the tumor progression and to test whether inflammasome-mediated IL-1β production is linked to tumor progression, we determine the IL-1β levels in spleen and tumor tissues of our B16-F10 melanoma models, at different days after the tumor induction, with Enzyme-linked immunosorbent assay (ELISA). Melanoma tumors from tumor-bearing mice and spleens from tumor-bearing and naïve mice (day 0) were collected, homogenized and assayed by IL-1β specific ELISA. As the tumor was growing, we observed elevated concentration of mature IL-1β in tumor microenvironments (Figure **3a**). In addition, in peripheral lymphoid organs, such as spleen, a gradual increase in IL-1β levels was reported in tumor spleens of melanoma-bearing mice compared with naïve animals and earlier stages of tumor development (Figure **3b**). Overall, these results demonstrate that mature IL-1β production is linked to tumor progression and suggest a role for IL-1β in promoting tumor growth.

3.1.3 Elevated NLRP3 signaling in DCs and MDSCs during melanoma progression

As is clear from the findings of this thesis so far, during tumor development myeloid cell compartment seems to be expanded and IL-1β is suggested to be correlated with tumor progression. Thus, we postulated that the increased levels of mature IL-1β in tumor microenvironment and peripheral lymphoid organs might be due to the NLRP3 inflammasome activation in myeloid cell compartment, and specifically in DCs and MDSCs. To address this, we examined the expression of NRLP3 inflammasome and pro-IL-1β in these two myeloid cell types in tumor and spleen tissues during tumor progression. However, due to the difference in the dilution of NLRP3 antibody used for intracellular staining between our laboratory and literature[170], we first assessed its appropriate concentration for all the experiments that will follow through a titration experiment, which showed the dilution 1:25 of NLRP3 antibody as the most reliable for detecting NLRP3 activation (Figure **4a**). Thus, single cell suspensions were prepared from tumor tissues and spleen on Day 8 and Day 15 after tumor induction, and then analyzed by FACS. Flow cytometric analysis demonstrated upregulated NLRP3 and pro-IL-1β expression in both tumor- and spleen-infiltrating DCs and MDSCs, already from early stages of tumor growth (Figure **4b,c**). We also examined the NLRP3 and pro-IL-1β expression at the endpoint of tumor progression according to our B16-F10 melanoma model (as described in material and methods), where mice presented notably increased tumor weight (Figure **5a**), and we find again increased NLRP3 and pro-IL-1β expression in tumor-infiltrating DCs and MDSCs (Figure **5b**). Regarding,

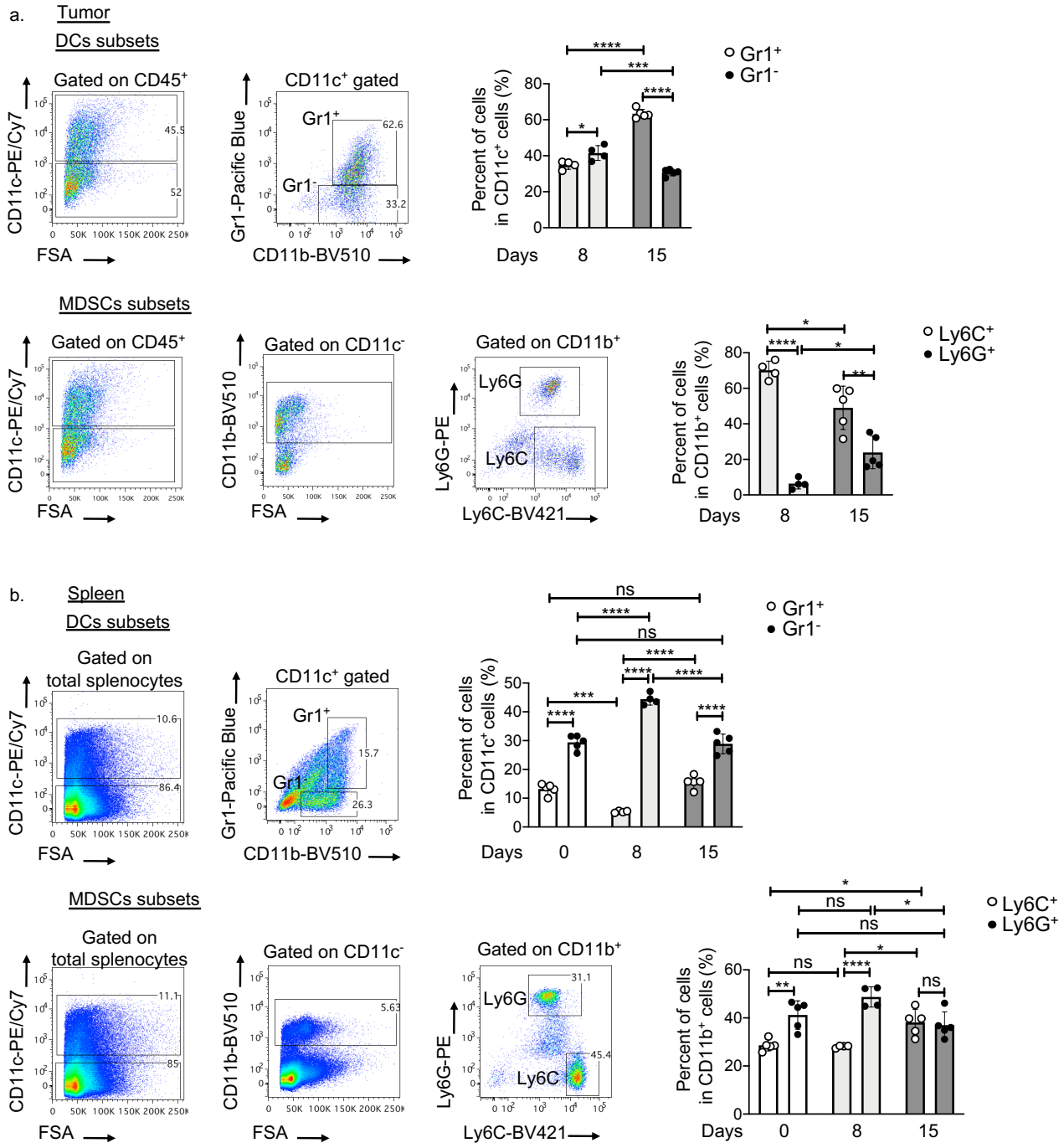

Figure 2. Infiltration of MDSC and DC cell subsets in tumor tissue and peripheral lymphoid organs in an induced murine melanoma tumor model. (a) Gating strategies and frequencies of intratumoral $CD11c^+CD11b^+Gr1^+$/ $CD11c^+CD11b^+Gr1^-$ and $CD11b^+Ly6C^{high}Ly6G^-$ / $CD11b^+Ly6C^{low}Ly6G^+$ cell subsets in their parent population. **(b)** Gating strategies and frequencies of spleen infiltrated $CD11c^+CD11b^+Gr1^+$/ $CD11c^+CD11b^+Gr1^-$ and $CD11b^+Ly6C^{high}Ly6G^-$/ $CD11b^+Ly6C^{low}Ly6G^+$ cell subsets in their parent population. Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

the corresponding populations in spleen, we observed expression of NLRP3 inflammasome pathway both in naive and tumor state (Figure **5c**). Interestingly, in naïve state we found that these two spleen-infiltrating myeloid populations have a notably higher expression of NLRP3 and pro-IL-1β compared to them of the endpoint tumor state (Figure **5c**). Together, these findings underscore the substantial expression of NLRP3 inflammasome pathway in MDSCs and DCs from naïve and melanoma-bearing mice.

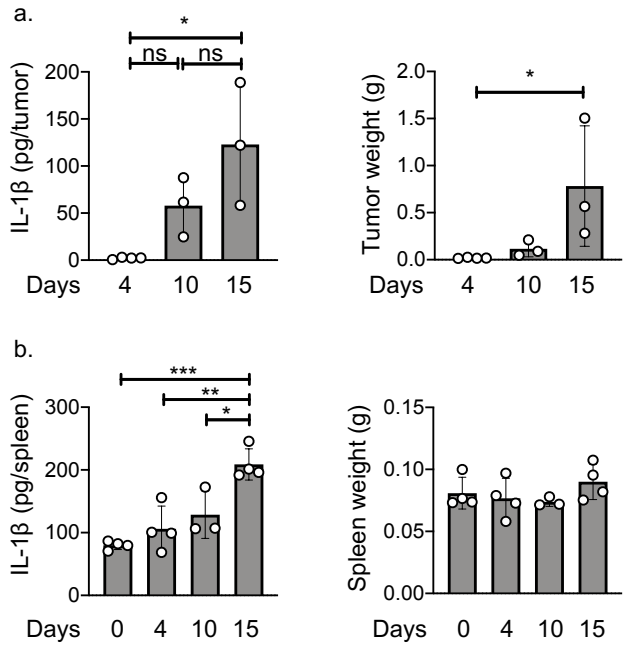

Figure 3. Increased IL-1β levels in TME and peripheral lymphoid organs during tumor development and progression. (a) IL-1β levels (pg/tumor) in tumor homogenates from B16.F10-inoculated mice and their respective tumor weights at indicated time points (day 4: n = 4; day 10: n = 3; day 15: n = 3). **(b)** IL-1β levels (pg/spleen) in spleen homogenates from B16.F10-inoculated mice and their respective spleen weights at indicated time points (day 0: n = 4; day 4: n = 4; day 10: n = 3; day 15: n = 4). Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biologically independent mouse samples.

Up to this point, we answered the question of whether DCs and MDSCs express NLRP3 and pro-IL-1β. The next question we sought to answer is whether these two myeloid cell populations express NLRP3 and pro-IL-1β more than any other of the cell population that infiltrate tumor and spleen tissues, and if so which of the two has greater expression and consequently stronger activation of the NLRP3 inflammasome signaling. To address these questions, we determined, through mean fluorescence intensity (MFI), the expression of NLRP3 and pro-IL-1β at different stages of tumor development again, in tumor- and spleen-infiltrating DCs and MDSCs and also in the rest $CD45^+$ leukocytes which are not DCs and MDSCs and infiltrate tumor and spleen tissues, and are characterized in this Master Thesis as $CD11c^-CD11b^-Gr1^-$ Double Negative (DN) population (as shown in gating strategy of Figure **1a,b**). Thus, from the cell populations which infiltrate tumor, a notably increase in NLRP3 inflammasome expression was observed in DCs, compared with MDSCs and rest tumor-infiltrating $CD45^+$ leukocytes that are included in the Double Negative population, both in earlier (Day 8) and later (Day 15) stages of tumor development (Figure **6a** & **7a**). Pro-IL-1β expression levels appeared to have a similar trend, which while on Day 8 were significantly higher in DCs, following those of MDSCs and Double Negative, on Day 15 endpoint pro-IL-1β levels of expression seemed to be equilibrated between DCs and MDSCs and quite reduced in the Double Negative population (Figure **6a** & **7a**). Interestingly, despite the low levels of NLRP3 expression in MDSCs, which were the same as those of the Double Negative population, their expression levels of pro-IL-1β were the same as those of DCs, which showed sharply higher expression of NLRP3 (Figure **7a**). In terms of peripheral lymphoid organs, analysis of spleen of tumor-bearing mice revealed a prominent increase in the NLRP3 expression levels of DCs, with immediately following being those of MDSCs and even lower those of the rest $CD45^+$ leukocytes, an expression pattern which seemed to occur both in earlier and later stages of tumor progression (Figure **6b** & **7b**). However, in the spleen, as opposed to the tumor, the expression of pro-IL-1β in DCs was shown to be higher than that of Double Negative population, but markedly decreased compared with that of MDSCs, a trend which appeared to happen throughout tumor development, as it was evident on both the 8^{th} and the 15^{th} day after melanoma tumor induction (Figure **6b** & **7b**). In addition, the expression profile of NLRP3 and pro-IL-1β that was observed in tumor spleen-infiltrating cell populations, was also noted in populations that infiltrated spleens of mice in naïve state, as DCs showed an upregulated NLRP3 expression compared with MDSCs, with the latter having notably increased pro-IL-1β expression levels (Figure **7c**). Collectively, these findings demonstrate that in tumor tissue, regardless of tumor progression stage, DCs show upregulated levels of NLRP3 expression compared to other populations. However, depending on the stage of tumor growth it is

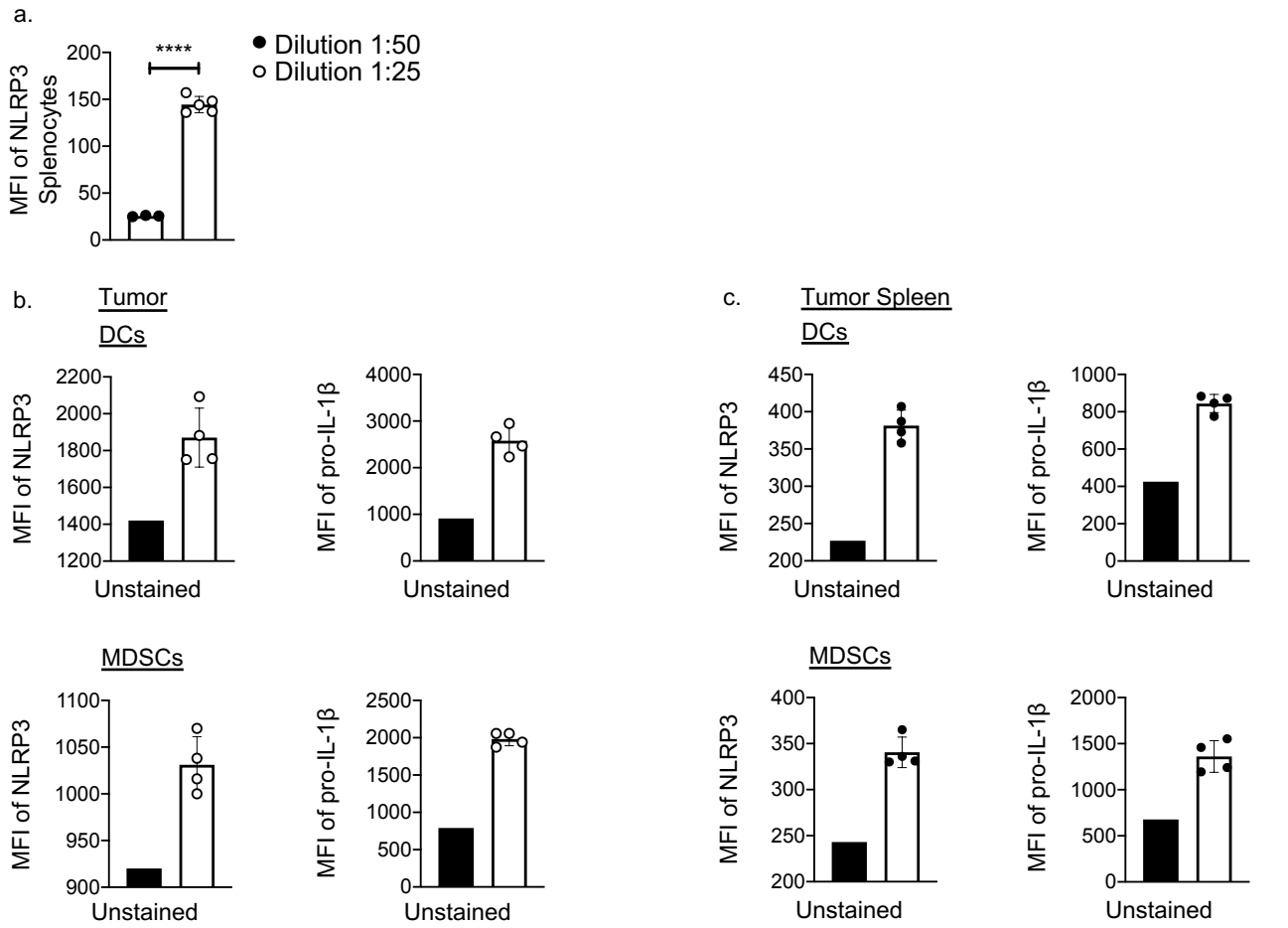

Figure 4. NLRP3 inflammasome and pro-IL-1β are highly expressed in DC and MDSC myeloid cell populations on the 8th day after melanoma tumor induction. (a) NLRP3 mean fluorescence intensity (MFI) of total splenocytes from B16.F10-inoculated mice using two different dilution of NLRP3 antibody (dilution 1:50: n=3; dilution 1:25: n=5). **(b)** MFI of NLRP3 and pro-IL-1β from tumor infiltrated DCs (n=4) and MDSCs (n=4). **(c)** MFI of NLRP3 and pro-IL-1β from spleen infiltrated DCs (n=4) and MDSCs (n=4). The unstained condition in all graphs emerged from the total staining excluding this of the protein of interest. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by Student's test (**a**). Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biologically independent mouse samples.

determined which population has the most elevated levels of pro-IL-1β expression and possibly contributes more to the secreted IL-1β detected in the TME. In spleen, both in naïve and in tumor state, the expression profile is more clear, as regardless of the stage of tumor development DCs have the highest levels of NLRP3 expression, while in MDSCs high pro-IL-1β expression is detected.

Next we aimed to examine, in addition to the expression levels of NLRP3 and pro-IL-1β in DCs and MDSCs populations, the levels of these two proteins via Flow cytometric analysis in the subsets of these two populations, and more particularly in $CD11c^{+}CD11b^{+}Gr1^{+}$ ($Gr1^{+}$) and $CD11c^{+}CD11b^{+}Gr1^{-}$ ($Gr1^{-}$) DCs cell subsets, as well as $CD11b^{+}Ly6C^{high}Ly6G^{-}$ ($Ly6C^{+}$) and $CD11b^{+}Ly6C^{low}Ly6G^{+}$ ($Ly6G^{+}$) MDSCs cell subsets. Thus, we found that in earlier stages of tumor development the expression levels of both NLRP3 and pro-IL-1β between tumor-infiltrating $Gr1^{+}$ and $Gr1^{-}$ DC subset population were not statistically different (Figure **8a**), whereas in the later stages $Gr1^{+}$ cells showed a prominent increase in NLRP3 expression compared with $Gr1^{+}$ cells, but again no difference in the levels of pro-IL-1β (Figure **9a**). Regarding the MDSC subset populations that infiltrate tumor, on the 8th day after melanoma tumor induction $Ly6G^{+}$ cells showed to have increased expression of NLRP3 and pro-IL-1β compared with $Ly6C^{+}$ cell subset population (Figure **8a**). However, this appeared to change on the 15th day of tumor growth, as $Ly6C^{+}$ cells reported to have upregulated NLRP3 expression, while pro-IL-1β expression levels continued to be elevated in the $Ly6G^{+}$ population (Figure **9a**). Moreover, we assessed NLRP3 and pro-IL-1β expression in DC and MDSC cell subsets of the spleens of naïve and tumor-inoculated mice. We observed similar expression patterns of NLRP3 and pro-IL-1β during tumor growth, both in the subsets of DCs and MDSCs. More specifically, $Gr1^{+}$ DC cell subset, in both earlier and later stages of tumor growth, found to have increased MFIs of NLRP3 and pro-IL-1β

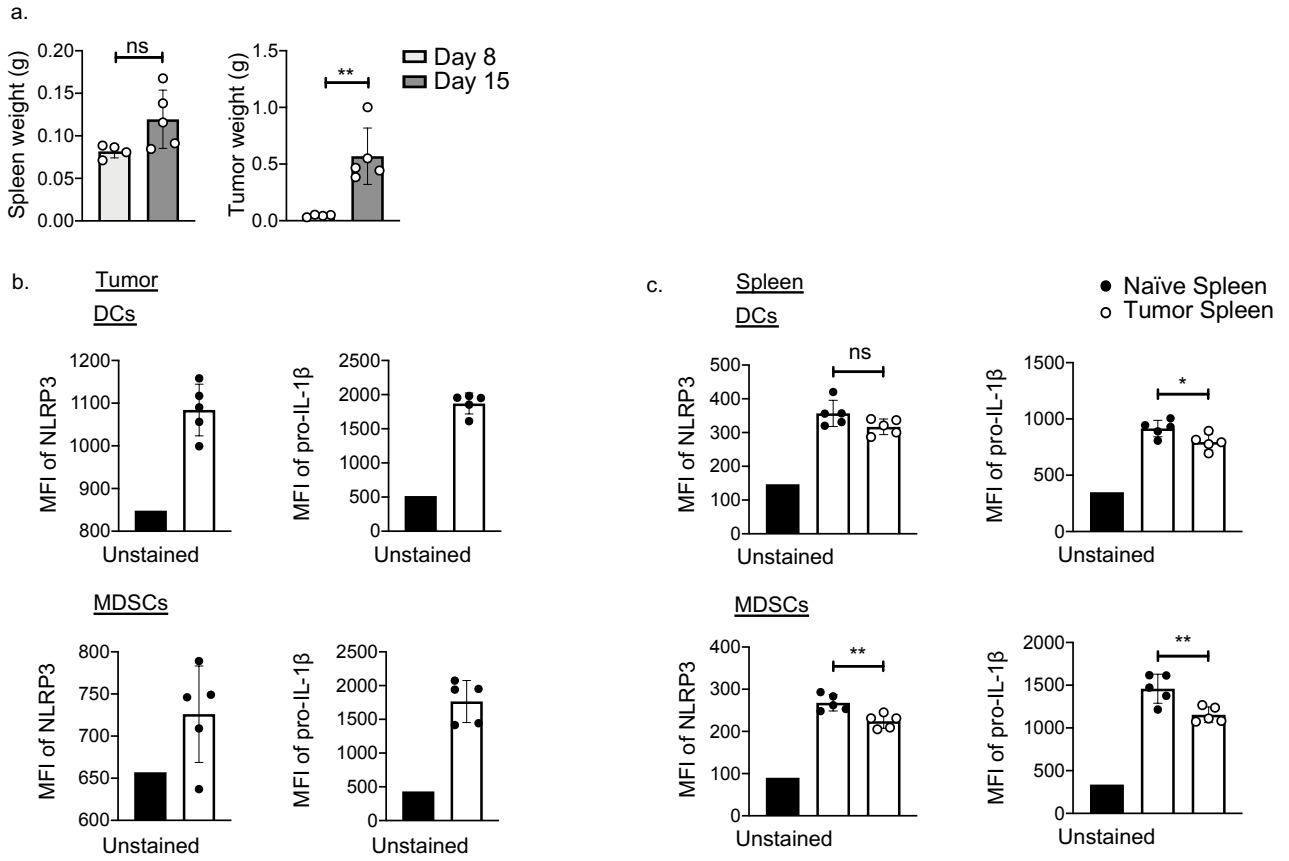

Figure 5. NLRP3 inflammasome and pro-IL-1β are highly expressed in DC and MDSC myeloid cell populations on the 15th day after melanoma tumor induction and naïve state. (a) Tumor and spleen weights of B16.F10-inoculated mice on Day 8 (n=4) and Day 15 (n=5) after melanoma induction. **(b)** MFI of NLRP3 and pro-IL-1β from tumor infiltrated DCs (n=5) and MDSCs (n=5). **(c)** MFI of NLRP3 and pro-IL-1β from DCs and MDSCs infiltrated in naïve (n=5) and tumor spleen (n=5). The unstained condition in all graphs emerged from the total staining excluding this of the protein of interest. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by Student's t-test (**a** and **c**). Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biologically independent mouse samples.

compared to $Gr1^-$, in the spleens of tumor-bearing mice (Figure **8b** and **9b**).The same is true about MDSC tumor spleen-infiltrating subsets, where $Ly6G^+$ cells showed to have an induction on NLRP3 and pro-IL-1β levels at both stages of tumor progression, when compared with the $Ly6C^+$ subset population (Figure **8b** and **9b**). A similar pattern of results was also found for DCs and MDSCs cell subsets, which infiltrate naïve spleen (Figure **9b**). Comparing the expression levels of NLRP3 and pro-IL-1β between the naïve and tumor state, we observed only in $Gr1^+$ DC cell subset a prominent decrease in levels of both proteins in the spleens of tumor-bearing mice, whereas their levels in $Gr1^-$ cells and MDSC cell subsets were not altered (Figure **9b**). Together, these results demonstrate the different levels of expression of NLRP3 inflammasome sensor protein and the production of pro-IL-1β in the different subsets of DCs and MDSCs myeloid cells during melanoma tumor development and progression, highlighting $Ly6G^+$ and $Gr1^+$ cells, as the subset populations of the MDSC and DC compartment, respectively, with elevated NLRP3 signaling.

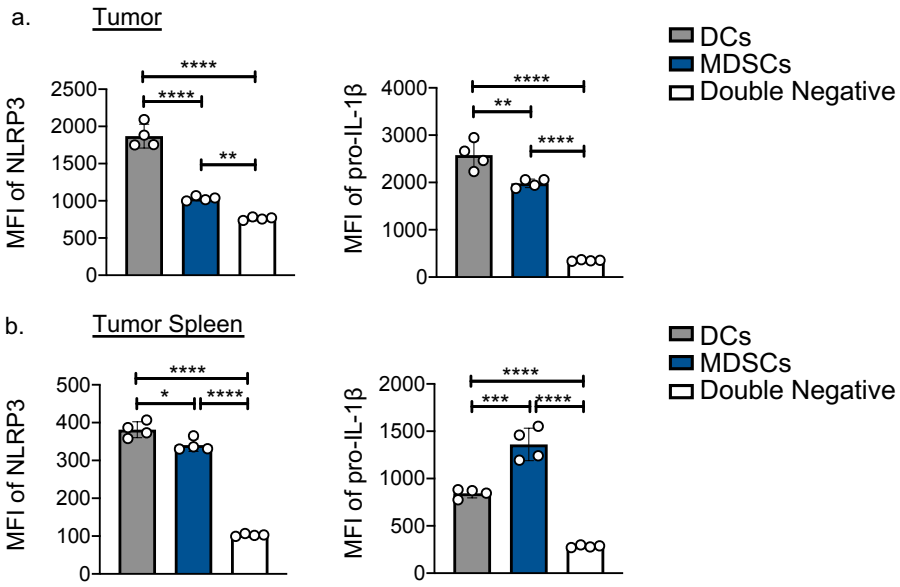

Figure 6. DCs and MDSCs are the myeloid cell subtypes with the highest expression of NLRP3 inflammasome and pro-IL-1β among the cell populations that infiltrate the tumor and spleen tissues on the 8th day after melanoma tumor induction. (a,b) $CD11c^+$ DCs (n=4), $CD11c^-CD11b^+Gr1^+$ MDSCs (n=4) and $CD11c^-CD11b^-Gr1^-$ Double Negative cells (n=4) infiltrated in tumor tissue **(a)**; and spleen of tumor-bearing mice **(b)**; were tested for expression of NLRP3 and pro-IL-1β. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biological independent mouse samples.

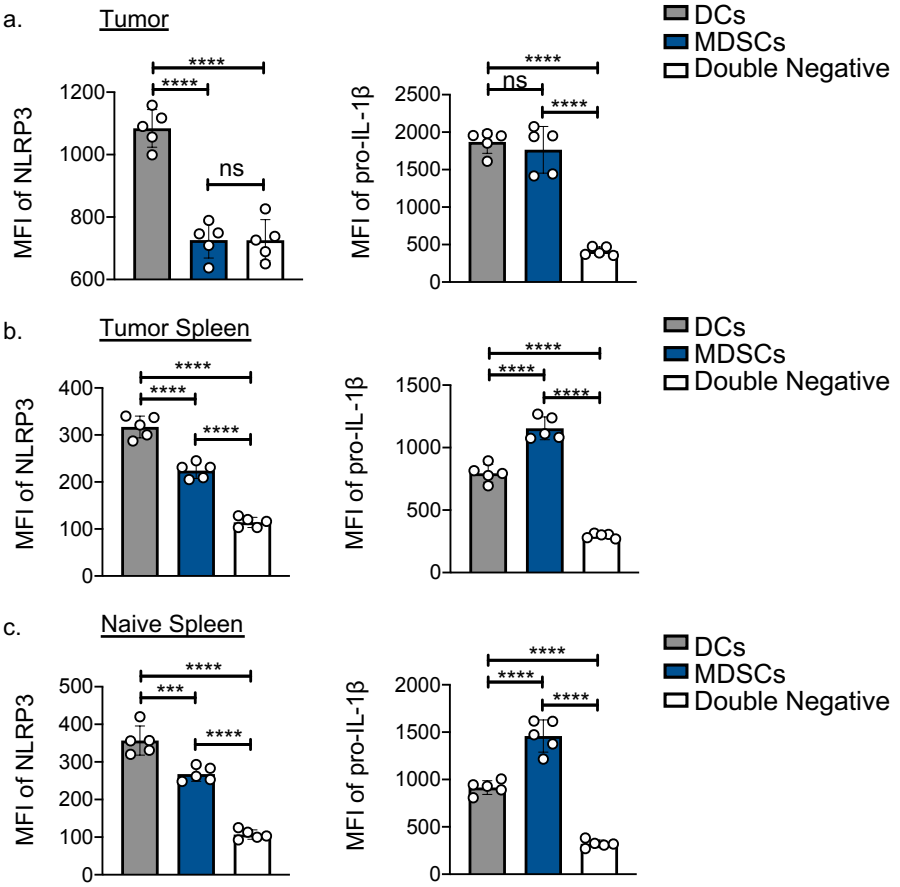

Figure 7. DCs and MDSCs are the myeloid cell subtypes with the highest expression of NLRP3 inflammasome and pro-IL-1β among the cell populations that infiltrate the tumor and spleen tissues on the 15th day after melanoma tumor induction and naïve state. (a,b,c) $CD11c^+$ DCs (n=5), $CD11c^-CD11b^+Gr1^+$ MDSCs (n=5) and $CD11c^-CD11b^-Gr1^-$ Double Negative cells (n=5) infiltrated in tumor tissue **(a);** and spleen of naïve **(b)**; and tumor-bearing mice **(c)**; were tested for the expression of NLRP3 and pro-IL-1β. Representative results from 2 independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biological independent mouse samples.

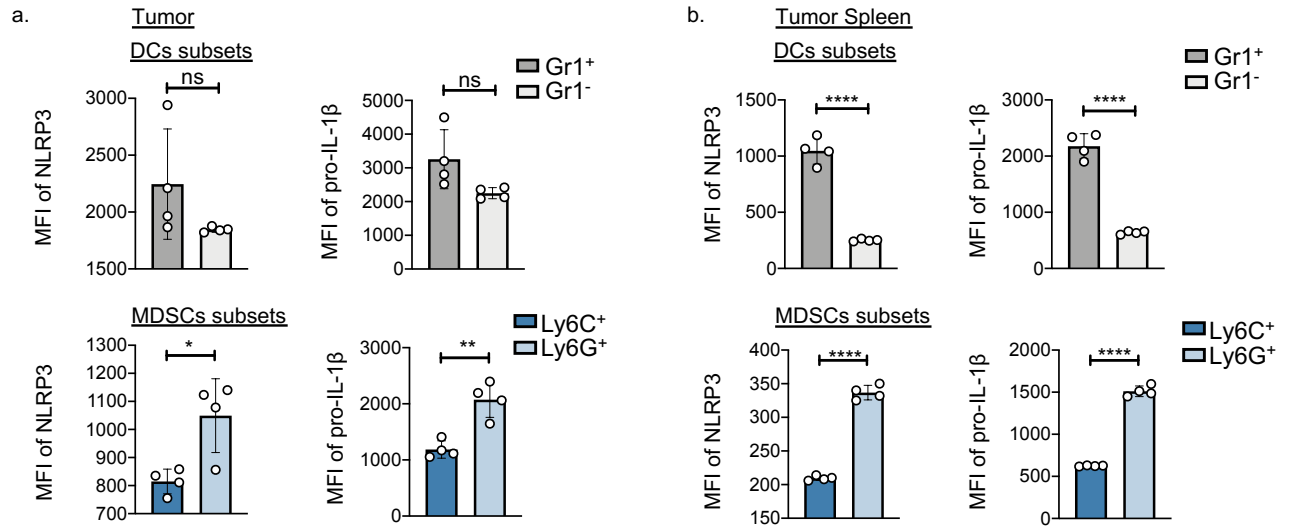

Figure 8. Expression of NLRP3 and pro-IL-1β in DC and MDSC cell subsets which infiltrated tumor and spleen tissue on the 8th day after melanoma tumor induction. (a,b) $CD11c^{+}CD11b^{+}Gr1^{+}$ (n=4) and $CD11c^{+}CD11b^{+}Gr1^{-}$ (n=4) DCs cell subsets, as well as $CD11b^{+}Ly6C^{high}Ly6G^{-}$ (n=4) and $CD11b^{+}Ly6C^{low}Ly6G^{+}$ (n=4) MDSCs cell subsets infiltrated in tumor tissue **(a)**; and spleen of tumor-bearing mice **(b)**; were tested for expression of NLRP3 and pro-IL-1β. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by Student's t-test. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biologically independent mouse samples.

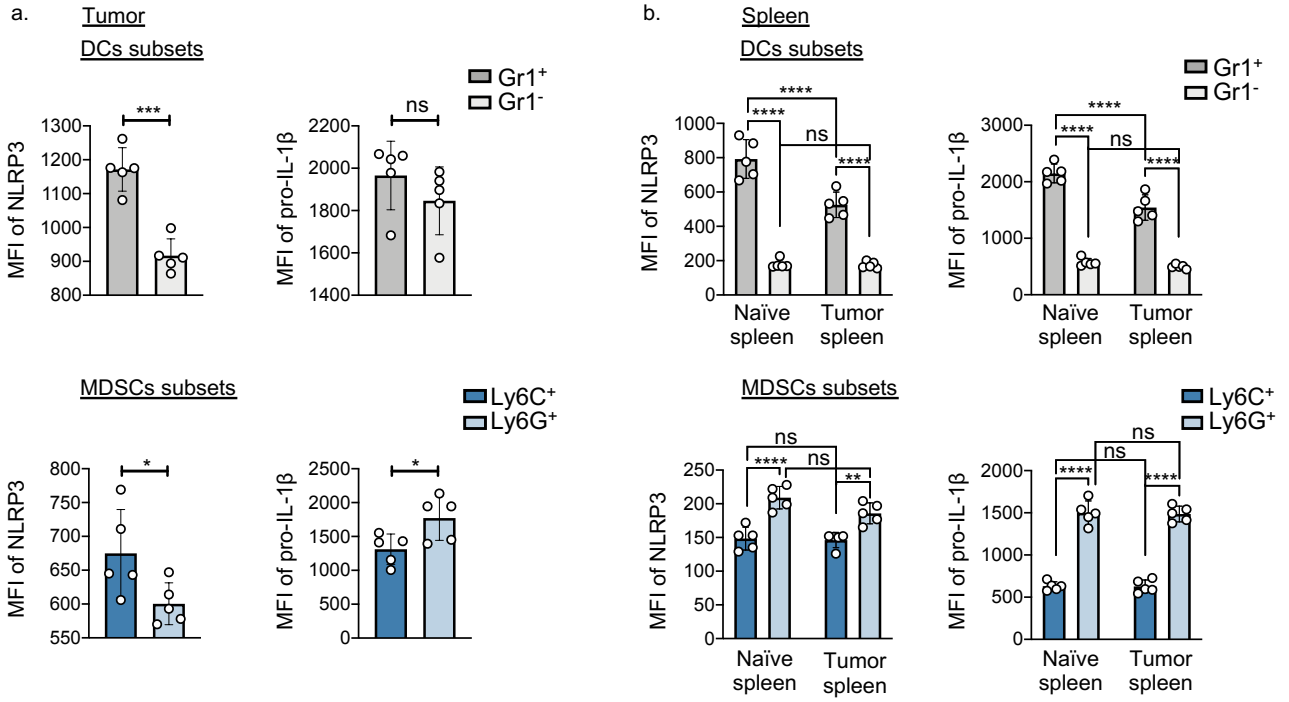

Figure 9. Expression of NLRP3 and pro-IL-1β in DC and MDSC cell subsets which infiltrated tumor and spleen tissue on the 15th day after melanoma tumor induction and naïve state. (a,b) $CD11c^{+}CD11b^{+}Gr1^{+}$ (n=4) and $CD11c^{+}CD11b^{+}Gr1^{-}$ (n=4) DCs cell subsets, as well as $CD11b^{+}Ly6C^{high}Ly6G^{-}$ (n=4) and $CD11b^{+}Ly6C^{low}Ly6G^{+}$ (n=4) MDSCs cell subsets infiltrated in tumor tissue **(a)**; and spleen of naïve and tumor-bearing mice **(b)**; were tested for expression of NLRP3 and pro-IL-1β. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by Student's t-test (**a**) and one-way ANOVA (**b**). Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001; n= biologically independent mouse samples.

3.1.4 LPS and ATP: a two-step inflammasome activation model that lead to pro-IL-1β and NLRP3 synthesis and the subsequent NLRP3 inflammasome-dependent IL-1β secretion in myeloid cells.

As described in great detail in the introduction of this Master thesis, the NLRP3 inflammasome is a multiprotein complex that is involved in IL-1β processing and activation in response to inflammatory stimuli. Most cell types are devoid of IL-1β, which is a cytokine that is produced predominantly by cells of the myeloid lineage, and only synthesize this in response to inflammatory stimuli (e.g. bacterial lipopolysaccharide (LPS)) but cleavage to its bioactive form in most cell types requires a secondary stimulus that activates the NLRP3 inflammasome complex. At present, in vitro experimental protocols examining NLRP3 inflammasome activation commonly include "priming" with a TLR agonist, such as lipopolysaccharide (LPS), and a second stimulus, such as adenosine triphosphate (ATP)[400, 401, 402, 403]. This two-signal model for IL-1β production is the predominant mode of activation in mouse macrophages[404, 405, 406]. Having shown up to this point in this Master Thesis, that the NLRP3 inflammasome signaling is elevated in myeloid cells, and more specifically that NLRP3 and pro-IL1-β are highly expressed in DCs and MDSCs, we next aimed to investigate whether the NLRP3 inflammasome in these myeloid cells is functional in response to cellular insults and evaluate whether its activation in these two populations obeys the two signal-model, which is well-studied in macrophages. For these reasons, we use LPS and ATP as stimuli to activate the NLRP3 inflammasome in myeloid cells in the present study.

But before we check the functionality of the NLRP3 inflammasome in myeloid cells, first we had to determine the right time point of priming signal with LPS and activation signal with ATP. In literature, there were many studies that used a wide range of incubation times with LPS and ATP, depending on the aim of the experiment but also with the cultured cells used. Thus, splenocytes isolated from naïve mice were primed with LPS for two different time points, 24 or 48 hours, and also stimulated for NLRP3 inflammasome activity with ATP for 30 minutes, 24 and 48 hours (Figure **10a**). After the activation time, splenocytes with flow cytometric analysis were analyzed for NLRP3 and pro-IL1-β expression in DC and MDSC populations (Figure **10b**). Regarding finding the right time point for the most effective priming of myeloid cells, we first observed elevated levels of NLRP3 and pro-IL1-β in MDSCs that were primed with LPS, compared with control cells (unstained and untreated cells), which appeared to happen both in 24 hours (Figure **10c**) and 48 hours (Figure **10d,e**) of LPS priming. NLRP3 expression levels showed not to be affected by the time of LPS priming, while levels of pro-IL1-β were more elevated at 48 hours LPS priming time point in MDSCs (Figure **10c-e**). Moreover, in DCs despite the fact that after the LPS priming NLRP3 levels did not show an increase compared with the control untreated cells, both in 24 hours (Figure **10c**) and 48 hours (Figure **10d,e**) of priming, the production of pro-IL1-β increased after the priming signal. As in MDSCs, so in DCs at 48 hours of priming the expressions levels of pro-IL1-β were higher (Figure **10c-e**). In our attempt to determine the appropriate time for the activation signal of NLRP3 inflammasome with the addition of ATP, we found that in both MDSCs and DCs the levels of NLRP3 were not modulated, whereas the levels of pro-IL1-β that were detected in MDSCs and DCs appeared to be decreased at 30 minutes (Figure **10d**), 24 hours (Figure **10c**) and 48hours (Figure **10e**). Comparing the levels of NLRP3 and pro-IL1-β in MDSCs and DCs between the different time points of priming and activation signals, we observed that the most upregulated levels correspond to 48 hours of priming and activation (Figure **10f**). However, in all three conditions we have significant expression of both NLRP3 and pro-IL1-β (Figure **10f**). Together, these findings demonstrate upregulated levels of NLRP3 and pro-IL1-β in MDSCs and DCs after LPS priming signal, at 24 and 48 hours. ATP activation signal does not modulate NLRP3 levels, while decreases pro-IL1-β levels that are detected in both myeloid populations, with the most increased levels belonging to the time point of 48 hours of ATP stimulation. However, due to the evident effect of ATP activation signal between 30 minutes and 24 hours, we decided for our following experiments to use as time for LPS priming signal the 24 hours, while as time for inflammasome activation the 1 hour.

As mentioned in the introduction, IL1-β is generated in the cytosol of the cells and its secretion is mediated via an unconventional secretory pathway which is independent from the conventional ER/Golgi trafficking route and the reason is the lack of signal peptide[306, 307]. However, due to the great uncertainty that exists in the literature concerning the use or not of Brefeldin A, a fungal metabolite that inhibit the protein transport through the collapse of Golgi apparatus, for the determination of intracellular levels of IL1-β, we assessed its effect on NLRP3 and pro-IL-1β levels. Thus, we found that Brefeldin A did not affect the expression levels of NLRP3 and pro-IL-1β in MDSCs and DCs, in both untreated (Figure **11a**) and LPS primed (Figure **11b**) splenocytes. However, where there may be a difference in the intracellular levels of IL1-β in the presence of Brefeldin A, is after the treatment with the ATP inflammasome activator, a stage in which the NLRP3 inflammasome becomes activated and the maturation cleavage of pro-IL1-β and the secretion of mature IL1-β occurs. Nevertheless, no significant difference was observed in the intracellular IL1-β levels after Brefeldin A treatment, as there was no further accumulation of IL1-β in the

cytosol of these cells compared with the cells that was not added in their supernatant (Figure **11c**). Collectively, these findings confirmed that IL1-β secretion is mediated independently of ER/Golgi trafficking route in MDSCs and DCs myeloid cells, so there is no need to use Brefeldin A in our next experiments.

LPS and ATP are commonly used to induce NLRP3 inflammasome expression in macrophages in vitro. For this reason, and having determined the required time points for priming and activation of NLRP3 inflammasome in myeloid cells, we examined whether LPS combined with ATP were able to modulate NLRP3 inflammasome expression and activation in primary cultured splenocytes. Splenocytes were gated on MDSC and DC compartment, and analyzed for the NLRP3 and pro-IL1-β expression levels (Figure **12a**). Both MDSCs and DCs constitutively expressed NLRP3 and pro-IL1β, as revealed by the untreated splenocytes (Figure **12b,c**). LPS treatment alone significantly upregulated NLRP3 and pro-IL1-β expression in MDSCs (Figure **12b**), but only the pro-IL1-β expression in DCs and not that of NLRP3, which was already elevated by the untreated condition (Figure **12c**). Moreover, ATP treatment alone did not modulate the levels of these two proteins both in MDSCs and DCs (Figure **12b,c**), whereas cells treated with ATP combined with LPS increased NLRP3 and pro-IL1-β in MDSCs (Figure **12b**), but did not altered their expression levels in DC population (Figure **12c**). Comparing the expression levels of NLRP3 and pro-IL1-β between the different populations of splenocytes, we observed that DCs have increased levels of NLRP3, which are not accompanied by increased production of pro-IL1-β, compared to MDSCs and double negative cells in the untreated condition (Figure **12d**). Furthermore, MDSCs showed enhanced levels of pro-IL1-β after the priming of splenocytes with LPS, whereas the NLRP3 and pro-IL1-β expression levels were markedly decreased in the double negative population in all culture conditions (Figure **12d**). In addition, we also examined whether tumor explant supernatant-treated (TES-treated) splenocytes modulated the NLRP3 inflammasome signaling in MDSCs and DCs myeloid cells, as it contains multiple DAMPs derived from necrotic cancer cells. To this end, flow cytometric analysis demonstrated no difference in NLRP3 and pro-IL-1β expression levels of MDSCs in TES-treated splenocytes, compared to the untreated cells (Figure **13a**), whereas a notably decrease in NLRP3 levels was observed in the DC population (Figure **13b**). Only in the presence of LPS stimulation the inflammasome signaling was induced in MDSCs (Figure **13a**), while in DCs no induction was observed in both levels of NLRP3 and pro-IL-1β (Figure **13b**). TES treatment did not modulate the expression profile of the different spleen-infiltrating populations, meaning that MDSCs still expressed prominent increased levels of pro-IL-1β, NLRP3 levels among MDSCs and DCs were not significantly different and the double negative population expressed markedly decreased of both proteins (Figure **13c**). Together, these findings demonstrate that MDSCs and DCs constitutively express NLRP3 and pro-IL-1β, and theur expression is upregulated by the stimulation with LPS, mainly in MDSCs that show prominent increase in pro-IL-1β, whereas ATP treatment alone fails to elevate the levels of these proteins in MDSCs and DCs. Moreover, TES treatment alone is unable to modulate the NLRP3 inflammasome signaling in MDSCs and DCs myeloid cells, and this happens only in the presence of LPS priming signal.

Finally, to provide direct evidence for the NLRP3 inflammasome expression and activation in MDSCs in vitro, we sorted MDSCs from the spleen of tumor-bearing mice and stimulated them with LPS and ATP (Figure **14a,b**). Flow cytometric analysis of NLRP3 and pro-IL-1β expression levels revealed that exposure of MDSCs to ATP alone did not altered their expression levels (Figure **14c**) and caused only modest secretion of IL-1β as determined by Elisa (Figure **14d**). Interestingly, ATP combined with LPS treatment increased NLRP3 and pro-IL-1β expression levels (Figure **14c**) and markedly induces the release of IL-1β (Figure **14d**). Overall, these findings provide evidence for NLRP3 inflammasome expression and activation in myeloid cells and more specifically in MDSCs, demonstrating that LPS upregulates NLRP3 and pro-IL-1β expression and ATP induces IL-1β secretion in LPS-primed MDSCs.

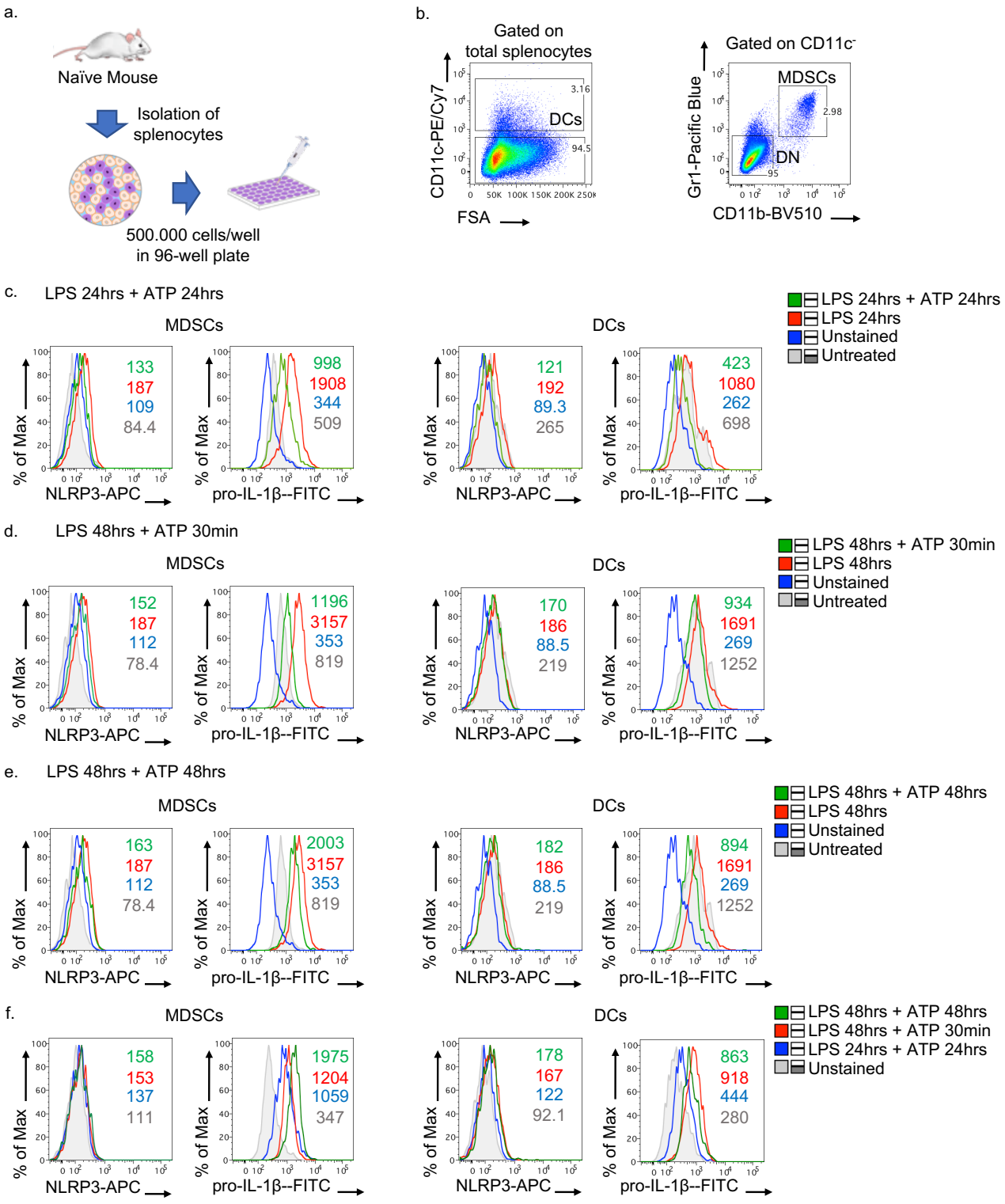

Figure 10. Expression of NLRP3 and pro-IL-1β in DCs and MDSCs after different stimulation time of splenocytes with LPS and ATP. **(a)** Splenocytes isolated from naïve mouse were seeded into 96-well plates at density 5x10^5 cells/well for inflammasome activation assays. Splenocytes were primed for 24 or 48 hours using LPS (1μg/ml). Cells were also stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator for the indicated time. After the NLRP3 inflammasome activation, stimulated splenocytes were collected for NLRP3 and pro-IL-1β expression analysis by flow cytometry. **(b)** Gating Strategy. **(c,d,e)** Histogram overlays of NLRP3 and pro-IL-1β expression (MFIs are shown) of CD11c$^-$CD11b$^+$Gr1$^+$ MDSCs and CD11c$^+$ DCs derived from in vitro cultures of splenocytes primed for 24 hours with LPS and stimulated for 24 hours with ATP **(c)**; primed for 48 hours with LPS and stimulated for 30 minutes with ATP **(d)**; primed for 48 hours with LPS and stimulated for 48 hours with ATP **(c)**, compared at each condition with the untreated cells, unstained cells for NLRP3 and pro-IL-1β, respectively, and cells which have been incubated with LPS for the indicated time. **(f)** Summary histogram overlays comparing the MFIs of NLRP3 and pro-IL-1β (MFIs are shown) of CD11c$^-$CD11b$^+$Gr1$^+$ MDSCs and CD11c$^+$ DCs upon priming and stimulation for the indicated time, using the unstained cells as control. Results from 1 experiment are shown.

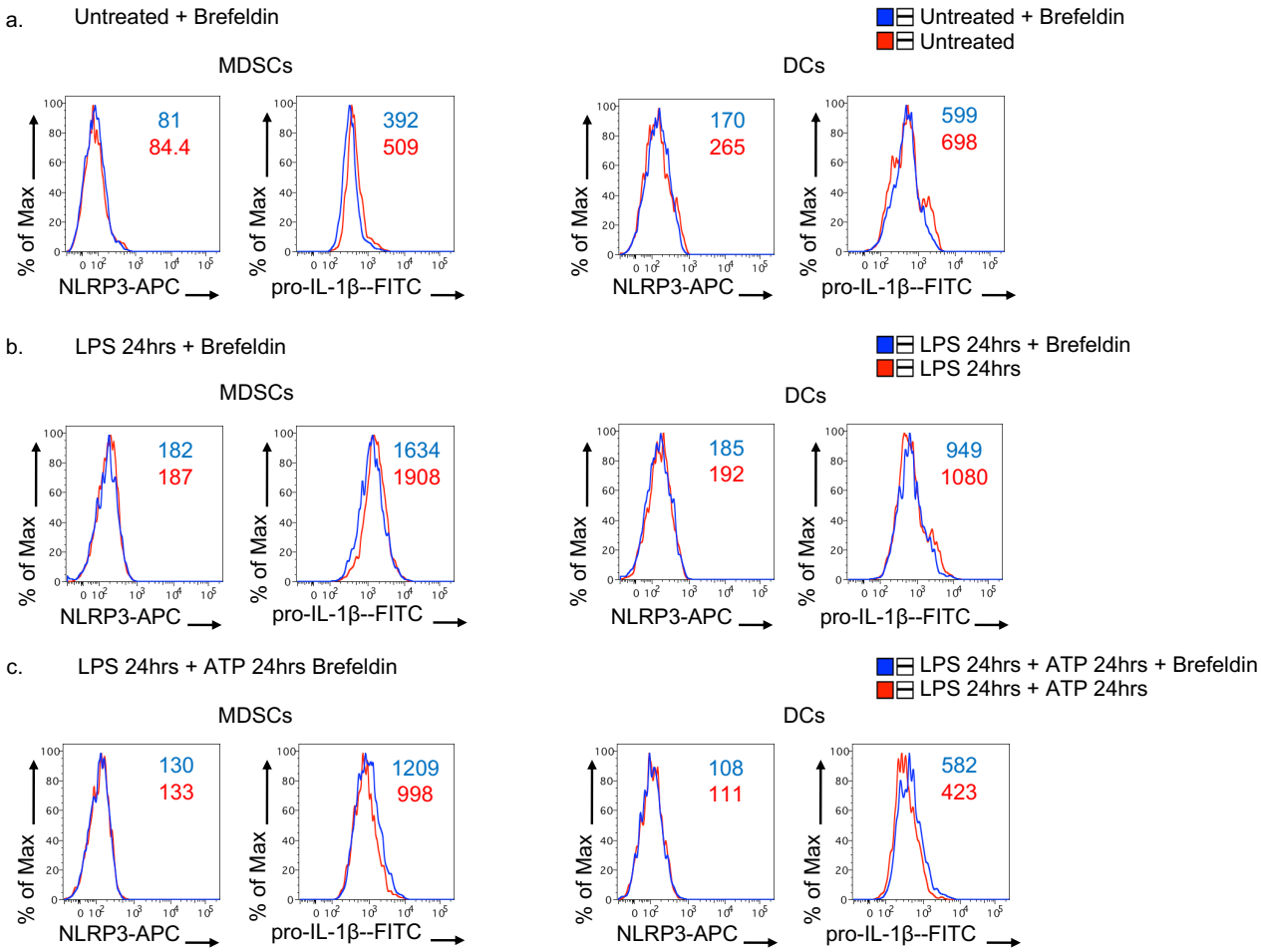

Figure 11. Intracellular levels of NLRP3 and IL-1β in DCs and MDSCs after stimulation of splenocytes in the presence of Brefeldin A. Splenocytes isolated from naïve mouse were seeded into 96-well plates at density $5x10^5$ cells/well for inflammasome activation assays. Splenocytes were primed for 24 hours using LPS (1μg/ml). Cells were also stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator for 24 hours. Four hours before the end of stimulation, Brefeldin A was added in the supernatant of cells. After the four-hour incubation time, stimulated splenocytes were collected for NLRP3 and pro-IL-1β expression analysis by flow cytometry. **(a,b,c)** Histogram overlays of NLRP3 and pro-IL-1β expression (MFIs are shown) of $CD11c^-CD11b^+Gr1^+$ MDSCs and $CD11c^+$ DCs derived from in vitro cultures of splenocytes that were not triggered with any stimulus in the presence or absence Brefeldin A **(a)**; primed for 24 hours with LPS in the presence or absence Brefeldin A **(b)**; primed for 24 hours with LPS and stimulated for 24 hours with ATP in the presence or absence Brefeldin A **(c)**. Results from 1 experiment are shown.

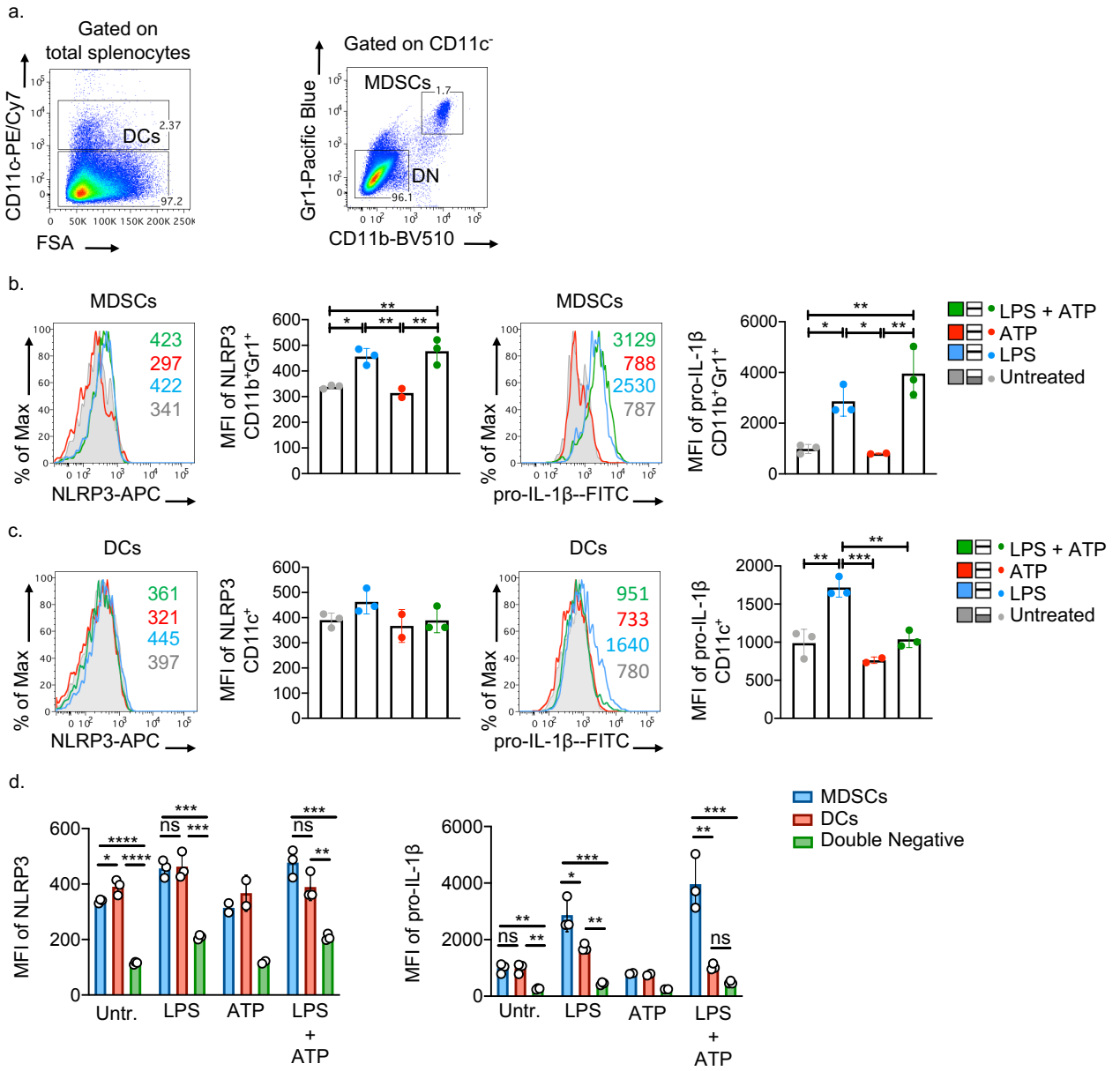

Figure 12. Expression of NLRP3 and pro-IL-1β in DCs and MDSCs after in vitro stimulation of splenocytes with LPS. Splenocytes isolated from three naïve mice (n=3, biologically independent mouse samples) were seeded in triplicate into 96-well plates at density 5x10^5 cells/well for inflammasome activation assays. Splenocytes were primed for 24 hours using LPS (1µg/ml). One hour before the end of priming, cells were stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator. One-hour post-NLRP3 inflammasome activation, stimulated splenocytes were collected for NLRP3 and pro-IL-1β expression analysis by flow cytometry. **(a)** Gating Strategy. **(b)** Representative histogram overlays of NLRP3 and IL-1β expression (MFIs are shown) and plots of NLRP3 and pro-IL-1β mean fluorescence intensity (MFI) of CD11c$^-$CD11b$^+$Gr1$^+$ MDSCs (n =3, biologically independent cell cultures) derived from in vitro cultures of splenocytes primed with LPS and stimulated with ATP. **(c)** Representative histogram overlays of NLRP3 and pro-IL-1β expression (MFIs are shown) and plots of NLRP3 and pro-IL-1β mean fluorescence intensity (MFI) of CD11c$^+$ DCs (n =3, biologically independent cell cultures) derived from in vitro cultures of splenocytes primed with LPS and stimulated with ATP. **(d)** Summary plots comparing the mean fluorescence intensity (MFI) of NLRP3 and pro-IL-1β of CD11c$^-$CD11b$^+$Gr1$^+$ MDSCs, CD11c$^+$ DCs and CD11c$^-$CD11b$^-$Gr1$^-$ Double negative cells (n =3, biologically independent cell cultures), upon priming and stimulation. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

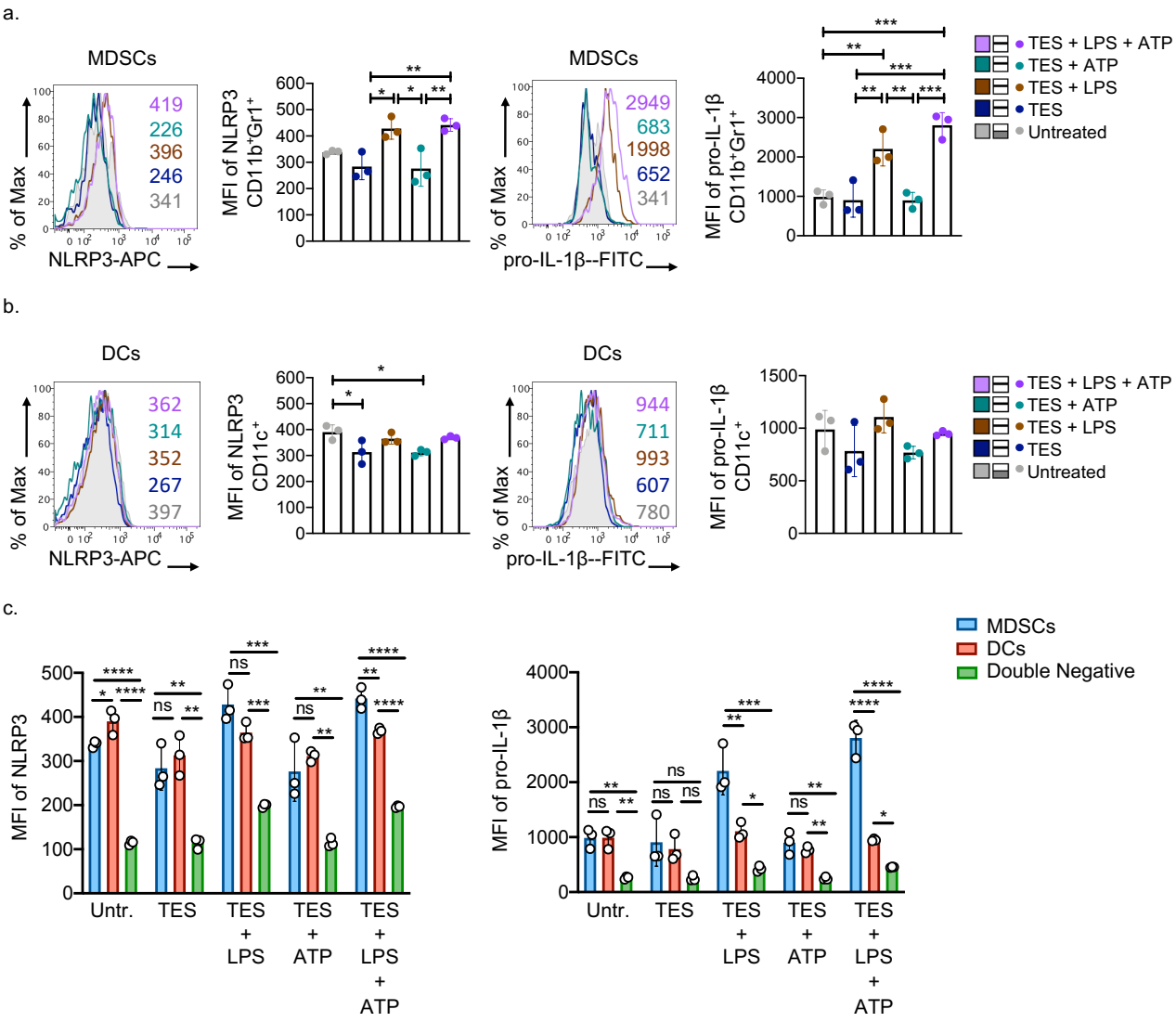

Figure 13. Expression of NLRP3 and pro-IL-1β in DCs and MDSCs after in vitro stimulation of splenocytes with TES. Splenocytes isolated from three naïve mice (n=3, biologically independent mouse samples) were seeded in triplicate into 96-well plates at density 5 x 10^5 cells/well for inflammasome activation assays. Splenocytes were primed for 24 hours using LPS (1µg/ml) and/or TES (20%). One hour before the end of priming, cells were stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator. One-hour post-NLRP3 inflammasome activation, stimulated splenocytes were collected for NLRP3 and pro-IL-1β expression analysis by flow cytometry. **(a)** Representative histogram overlays of NLRP3 and IL-1β expression (MFIs are shown) and plots of NLRP3 and pro-IL-1β mean fluorescence intensity (MFI) of $CD11c^-CD11b^+Gr1^+$ MDSCs (n =3, biologically independent cell cultures) derived from in vitro cultures of splenocytes primed with LPS and/or TES and stimulated with ATP. **(b)** Representative histogram overlays of NLRP3 and pro-IL-1β expression (MFIs are shown) and plots of NLRP3 and pro-IL-1β mean fluorescence intensity (MFI) of $CD11c^+$ DCs (n =3, biologically independent cell cultures) derived from in vitro cultures of splenocytes primed with LPS and/or TES and stimulated with ATP. **(c)** Summary plots comparing the mean fluorescence intensity (MFI) of NLRP3 and pro-IL-1β of $CD11c^-CD11b^+Gr1^+$ MDSCs, $CD11c^+$ DCs and $CD11c^-CD11b^-Gr1^-$ Double negative cells (n =3, biologically independent cell cultures), upon priming and stimulation. Data are shown as mean (±S.D.). Representative results from 2 independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

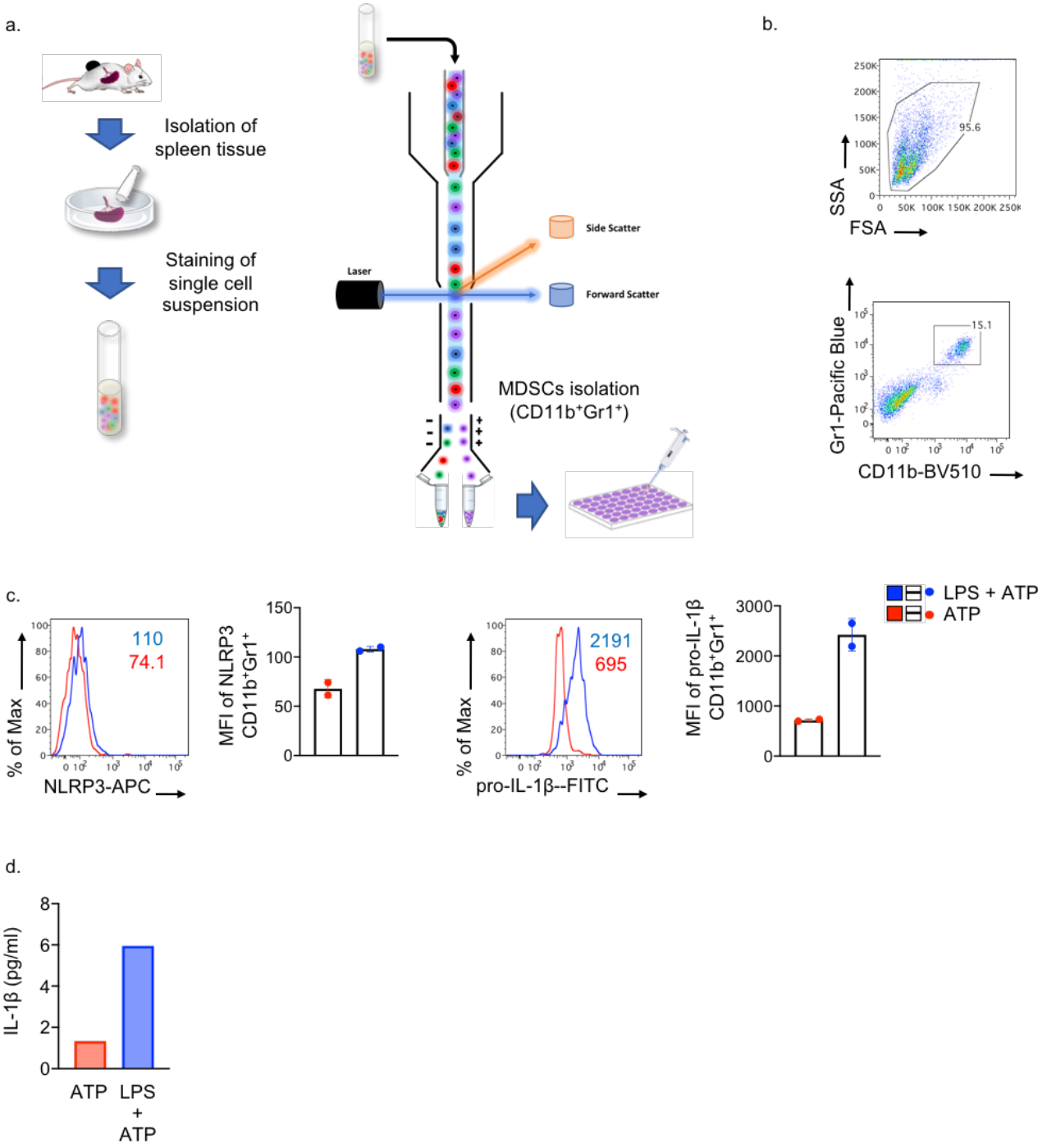

Figure 14. LPS and ATP upregulates NLRP3 and pro-IL-1β expression and induces IL-1β secretion in sorted MDSCs. (a) Scheme of MDSCs isolation procedure. Day 15 tumor-bearing mice (n=2, biologically independent mouse samples) were euthanized and spleen tissue was isolated for single cell suspension and sorting of MDSCs. Then, sorted MDSCs were seeded in duplicate into 96-well plate at density 10 x 10^4/well and were primed for 24 hours using LPS (1μg/ml). One hour before the end of priming, cells were stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator. One-hour post-NLRP3 inflammasome activation, stimulated splenocytes were collected for NLRP3 and pro-IL-1β expression analysis by flow cytometry and supernatants were also collected for IL-1β Elisa. **(b)** Gating strategy for analyzing the NLRP3 and pro-IL-1β expression from a total pure MDSC population. **(c)** Representative histogram overlays of NLRP3 and pro-IL-1β expression (MFI is shown) and plots of NLRP3 and pro-IL-1β mean fluorescence intensity (MFI) of sorted $CD11c^-CD11b^+Gr1^+$ MDSCs (n =2, biologically independent cell cultures) primed with LPS and stimulated with ATP. **(d)** IL-1β levels (pg ml^{-1}) in the supernatants of stimulated sorted $CD11c^-CD11b^+Gr1^+$ MDSCs (n =2, biologically independent cell cultures) determined by ELISA. Representative results from 2 independent experiments are shown.

3.2 Effects of NLRP3 inflammasome on tumor growth and anti-tumor immunity

3.2.1 NLRP3 inflammasome deficiency attenuates tumor growth and modulates anti-tumor immune responses

To determine the impact of inflammasome activity on tumor progression and anti-tumor immunity, we examined melanoma tumor growth in NLRP3 inflammasome deficient mice ($NLRP3^{-/-}$). $NLRP3^{-/-}$ were generated by the disruption of the NLRP3 gene by an EGFP cassette, which was inserted in frame with the ATG of exon 2 (Figure **15a**). qPCR (Figure **15b**) and flow cytometric analysis (Figure **15c,d**), which showed the increased percentages of $EGFP^+$ $CD11c^-CD11b^+Gr1^+$ MDSCs (Figure **15c**) and $CD11c^+$ DCs (Figure **15d**) in tumor and spleen tissue of $NLRP3^{-/-}$ mice compared with Wild Type (WT) mice, confirmed our NLRP3 inflammasome knock out mouse model. Moreover, to further confirm the NLRP3 inflammasome deficiency we isolated splenocytes from naïve WT and $NLRP3^{-/-}$ mice and after LPS and ATP stimulation we determined the levels of IL-1β in the supernatants of cultured cells. We observed, that $NLRP3^{-/-}$ splenocytes showed minimal levels of secreted IL-1β, whereas splenocytes from WT mice secreted a great amount of IL-1β, confirming thus the deficiency of NLRP3 inflammasome and the NLRP3-dependent IL-1β production (Figure **15e**).

To address the precise role of NLRP3 inflammasome in tumor growth and anti-tumor immunity, WT and $NLRP3^{-/-}$ mice were challenged with B16.F10-induced melanoma tumor for 19 days. Interestingly, the B16.F10-inoculated $NLRP3^{-/-}$ mice presented notably reduced tumor volume and tumor weight, compared with WT animals (Figure **16a**). Paradoxically, no difference was found in the IL-1β levels in tumors of $NLRP3^{-/-}$ and WT tumor-bearing animals (Figure **16b**), accompanied by the frequencies and numbers of tumor-infiltrating $CD45^+$ leukocytes which were not altered between the two groups (Figure **16c**). Thus, we next asked how the anti-tumor immune response was affected during tumor development by the deficiency of NLRP3 inflammasome. Regarding the myeloid cell compartment, we observed $CD11c^+$ DCs to have a tendency to increase on NLRP3-deficient mice, which, however was not statistically significant (Figure **17a**). Surprisingly, $NLRP3^{-/-}$ mice exhibited prominent increased frequencies in the $CD11c^+CD11b^+Gr1^+$ ($Gr1^+$) and markedly decreased frequencies in the $CD11c^+CD11b^+Gr1^-$ ($Gr1^-$) subset of DC population (Figure **17a**). Moreover, MDSC population showed not to be altered by the deficiency of NLRP3 inflammasome, as no differences were found in the frequencies and numbers of intratumoral MDSCs and their subsets between the two groups (Figure **17b**). Analysis of tumor-infiltrating lymphoid cell compartment revealed an increasing trend of frequencies and numbers of $CD4^+$ and $CD8^+$ T cells in $NLRP3^{-/-}$ mice, which was not statistically significant (Figure **18a**). In addition, numbers and frequencies in total tumor cells of $CD4^+Foxp3^+$ Treg and $CD4^+Foxp3^-$ Teff showed a non-statistically significant increasing trend in $NLRP3^{-/-}$ mice, whereas their frequencies in $CD4^+$ T cells were not altered compared with control animals (Figure **18a**). Furthermore, the frequencies and numbers of $NK1.1^+$ cells significantly increased in the tumors of $NLRP3^{-/-}$ mice (Figure **18b**).

We also analyzed peripheral lymphoid organs and more specifically the spleen tissue. Accordingly, B16.F10-inoculated $NLRP3^{-/-}$ mice presented slightly reduced spleen weights (Figure **19a**), whereas the IL-1β levels in the whole tissue were not altered between the two groups (Figure **19b**). Analysis of spleen-infiltrating cells of the two groups did not demonstrate differences in the frequencies of $CD11c^+$ DCs and their subsets (Figure **19c**). Surprisingly, despite the fact that no important changes in the frequencies of spleen-infiltrating MDSCs were monitored in tumor-bearing WT and $NLRP3^{-/-}$ animals, $CD11b^+Ly6C^{high}Ly6G^-$ ($Ly6C^+$) MDSC cell subset was significantly enriched in the spleen of $NLRP3^{-/-}$ mice, whereas the frequencies of $CD11b^+Ly6C^{low}Ly6G^+$ ($Ly6G^+$) MDSC cell subset were notably decreased in the spleen of these mice, compared with the control animals (Figure **19d**).

Overall, these findings suggest a tumor-promoting role for the NLRP3 inflammasome, as its deficiency impairs tumor development and associates with alterations of immune cell infiltration into melanoma tumors and peripheral lymphoid organs. Importantly, despite the same frequencies of tumor-infiltrating $CD45^+$ leukocytes observed between the two groups, in the myeloid compartment only the DC cell subsets showed modulated frequencies, with the $CD11c^+CD11b^+Gr1^+$ ($Gr1^+$) cells to be enriched and the $CD11c^+CD11b^+Gr1^-$ ($Gr1^-$) cells to be decreased in $NLRP3^{-/-}$ animals, whereas no alterations in the frequencies of DCs and in MDSC compartment were noted. In the lymphoid cell compartment, the NK cells showed a prominent increase in $NLRP3^{-/-}$ mice, whereas the T cell populations paradoxically demonstrated the same frequencies between the two different groups. In spleens of these animals, the myeloid context remained the same but, in contrast to tumor tissue, here the modulated frequencies were noted in the MDSC compartment, where $CD11b^+Ly6C^{high}Ly6G^-$ ($Ly6C^+$) cells were increased and $CD11b^+Ly6C^{low}Ly6G^+$ ($Ly6G^+$) were reduced in $NLRP3^{-/-}$ animals.

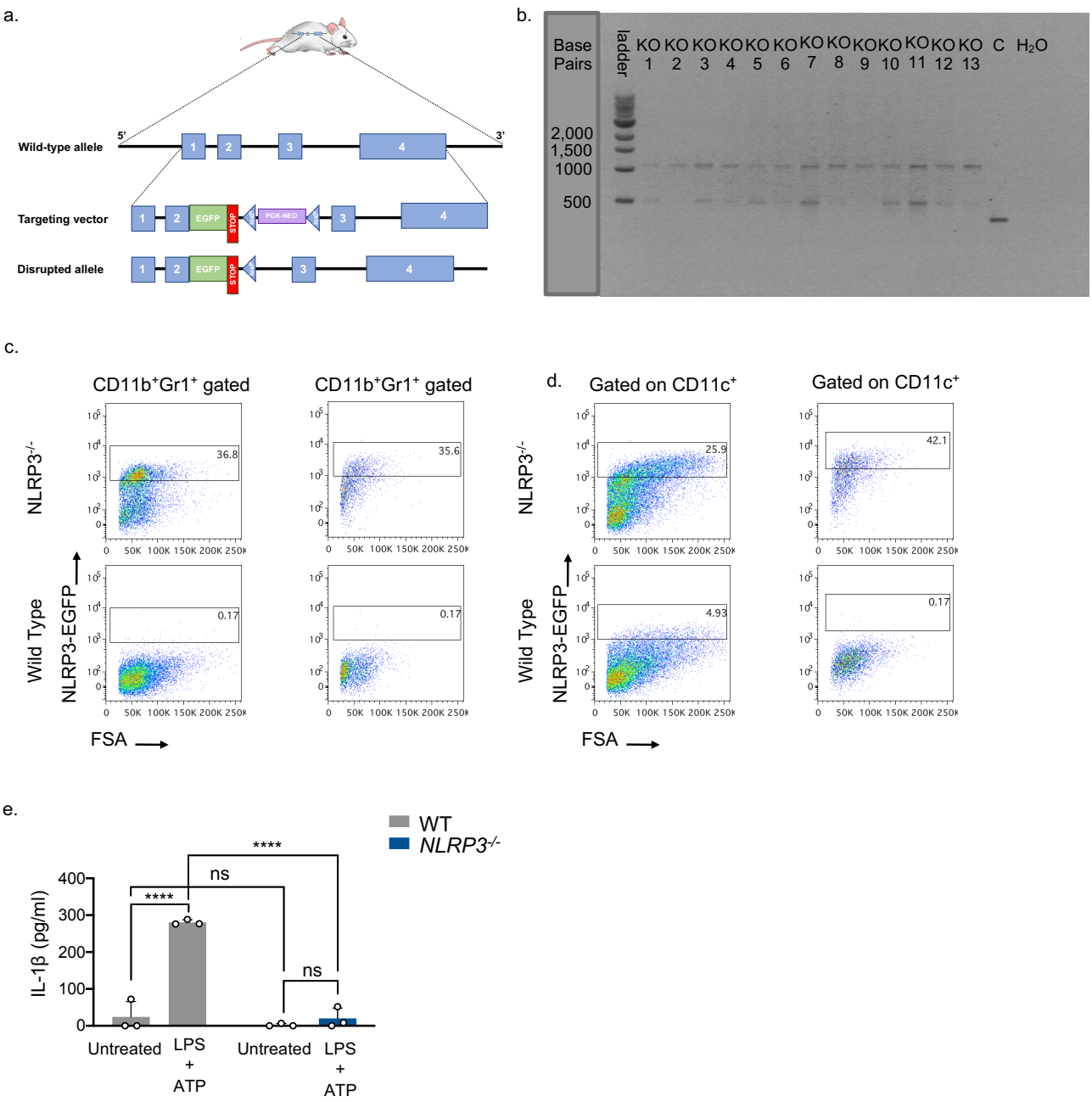

Figure 15. NLRP3 inflammasome knock out mouse model. (a) For the disruption of the mouse NLRP3 gene and the generation of this transgenic mouse line, an EGFP cassette was inserted in frame with the start codon of exon 2, which is followed by a SV40 poly(A) tail that promotes transcript degradation and is a terminator sequence that signals the end of a transcriptional unit, thereby following the disruption of the NLRP3 gene. A PGK-neo selection cassette was also inserted int the intron 2, which was flanked by 2 loxP sites and was deleted by the backcrossing of the mice, with the targeting vector, with a Cre-expressing strain (C57BL/6J) resulting in a NLRP3-/- mice on a C57BL/6J background. **(b)** PCR Genotyping of all the NLRP3$^{-/-}$ mice that have been used in the experiments of this master thesis. As control mice (C) have been used Atg5$^{fl/fl}$ mice. **(c,d)** Percentages of EGFP^{+} cells in intrasplenic **(c, left)** and intratumoral **(c, right)** MDSCs; and intrasplenic **(d, left)** and intratumoral **(d, right)** DCs. **(e)** IL-1β levels (pg ml^{-1}) in the supernatants of untreated and stimulated wild type and NLRP3$^{-/-}$ splenocytes (n =3, biologically independent cell cultures) determined by ELISA. Splenocytes isolated from wild type and NLRP3$^{-/-}$ naïve mice (n=3, biologically independent mouse samples) were seeded into 96-well plates at density 5x10^{5} cells/well. Splenocytes were primed for 24 hours using LPS (1μg/ml). One hour before the end of priming, cells were stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator. One-hour post-NLRP3 inflammasome activation, supernatants were collected for IL-1β Elisa. Data are shown as mean (±S.D.). Representative results from 3 (**c,d**) independent experiments are shown. Statistical significance was obtained by one-way ANOVA. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

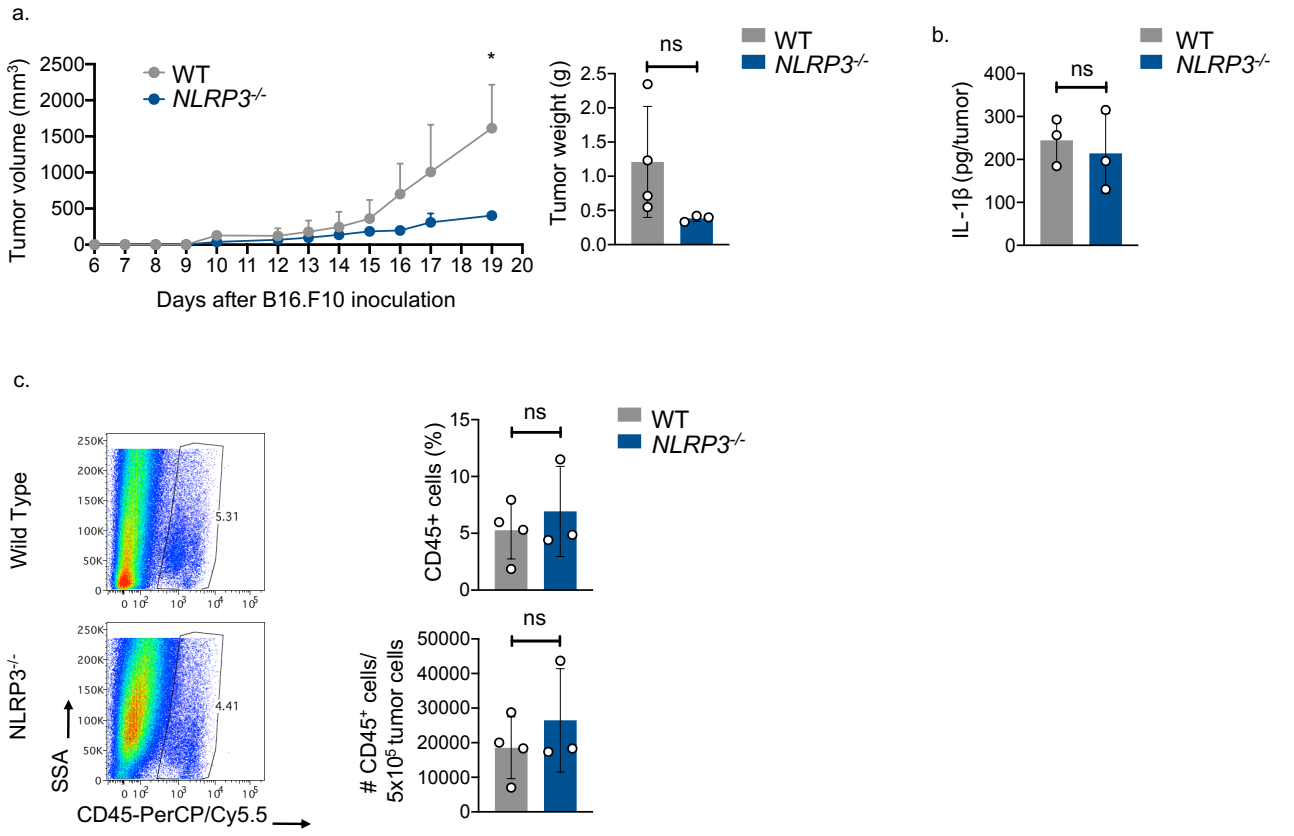

Figure 16. NLRP3 inflammasome deficiency inhibits tumor growth. (a) Tumor volume curve of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 9-19 days after B16.F10 inoculation and tumor weight on day 19. **(b)** IL-1β levels (pg/tumor) in tumor homogenates from B16.F10-inoculated Wild type (n=3) and NLRP3$^{-/-}$ (n=3) mice on day 19. **(c)** Representative FACS plots, frequencies of intratumoral CD45^{+} cells in total tumor cells and numbers per 5×10^{5} tumor cells of CD45^{+} cells of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation. Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by unpaired Student's t test. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

3.2.2 MCC950 is a potent inhibitor of NLRP3 inflammasome activation that abrogates IL-1β in splenocytes stimulated with NLRP3 inflammasome activators

In 2001 a number of diarylsulfonylurea-containing compounds were identified as novel IL-1β processing inhibitors by Perregaux et al.[407], which have been described to inhibit LPS plus ATP-induced release of mature IL-1β. In this Master Thesis we investigated one of these, which is termed MCC950 and is a potent selective NLRP3 inhibitor. Having observed from our knock out model that the inhibition of NLRP3 inflammasome results in tumor regression, our next goal was to determine if blockage of NLRP3 inflammasome affects myeloid cell function, and more specifically MDSC suppressive function, in vitro. Thus, for our functional experiments we first aimed to verify the potency of MCC950 to inhibit the NLRP3 inflammasome in our experimental conditions. The effect of MCC950 on the NLRP3 inflammasome activation was tested in primary splenocytes from naïve mice. Cells were first primed with LPS, then pretreated with MCC950 for two different time points and lastly stimulated with the NLRP3 stimulus ATP. To evaluate whether the MCC950 inhibitor had a cytotoxic effect on splenocytes in the 2μM concentration and for the time points that we have used, cells were collected for the estimation of the degree of apoptosis with 7-AAD viability staining solution and analyzed by flow cytometry. Our results showed that treating cells with 2 μM of MCC950 time-dependently did not affect the viability of the cells, because there are no differences in the numbers of cells undergoing cell death, as assessed by the 7-AAD staining in splenocytes treated with and without the MCC950 inhibitor (Figure **20a**). However, increased apoptotic cell death was evident in the untreated splenocytes, which treated neither with LPS and ATP stimulation nor MCC950 inhibitor (Figure **20a**). Moreover, splenocytes gated on the MDSC and DC compartment revealed that neither the viability of MDSC (Figure **20b**) nor the viability of DC (Figure **20c**) population was affected by the MCC950 time-dependently treatment. In addition, to verify the potency of MCC950 to inhibit the NLRP3 inflammasome, we collected the supernatants of the cultured splenocytes and we determined the IL-1β levels by Elisa. We observed that treating cells with 2μM concentration

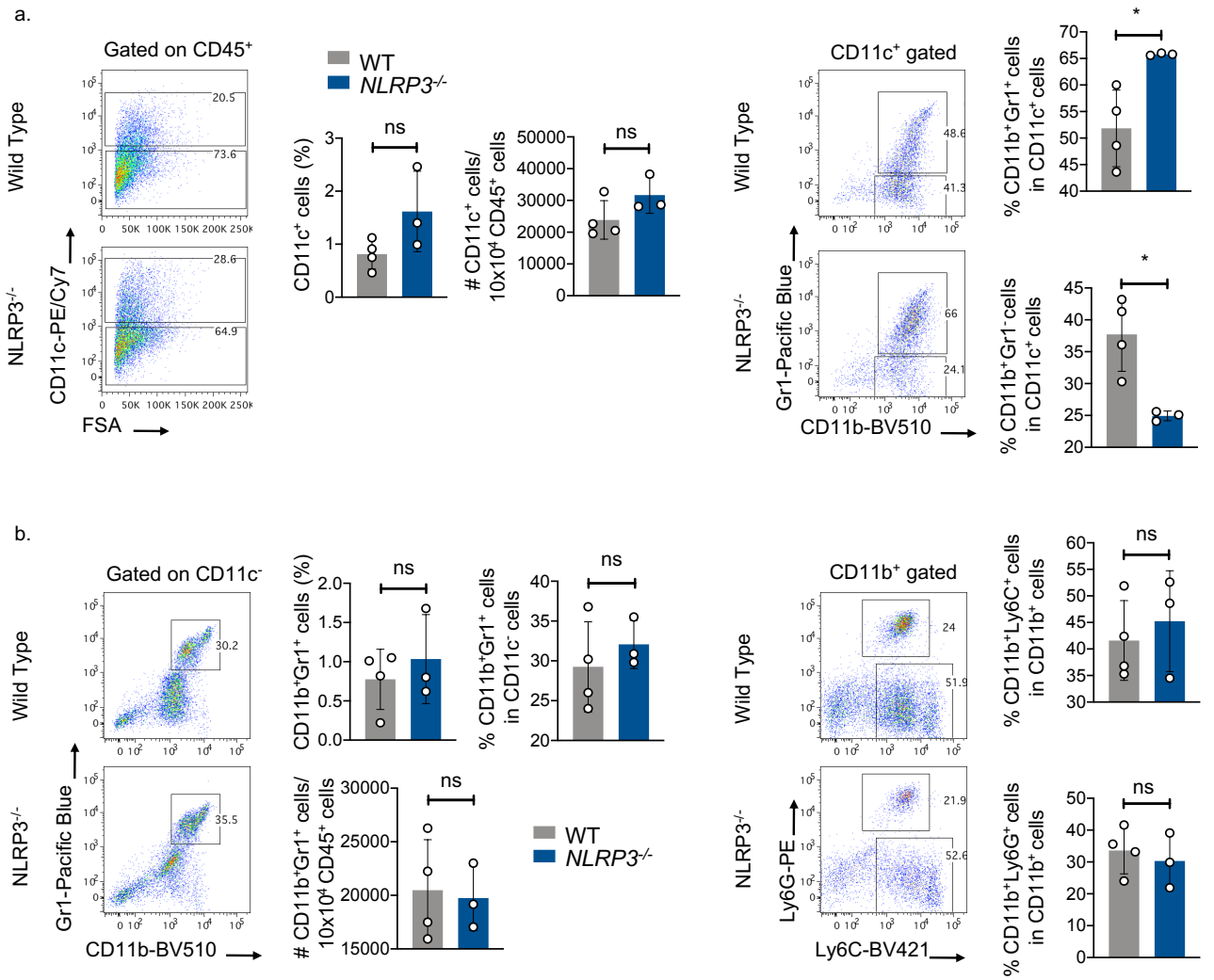

Figure 17. Effect of the NLRP3 inflammasome deficiency in tumor-infiltrated myeloid cell compartment. (a) Representative FACS plots, frequencies of intratumoral DCs ($CD11c^+$) in total tumor cells and their numbers per 10×10^4 $CD45^+$ cells of Wild type (n=4) and $NLRP3^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**a, left**); Representative FACS plots and frequencies of intratumoral $CD11c^+CD11b^+Gr1^+$ ($Gr1^+$) and $CD11c^+CD11b^+Gr1^-$ ($Gr1^-$) DC subsets in DC cell population (percentages of gated populations) of Wild type (n=4) and $NLRP3^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**a, right**). **(b)** Representative FACS plots, frequencies of intratumoral MDSCs ($CD11c^-CD11b^+Gr1^+$) in total tumor cells and in $CD11c^-$ cells (percentages of gated populations); and their numbers per 10×10^4 $CD45^+$ cells of Wild type (n=4) and $NLRP3^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**b, left**); Representative FACS plots and frequencies of intratumoral $CD11b^+Ly6C^{high}Ly6G^-$ ($Ly6C^+$) and $CD11b^+Ly6C^{low}Ly6G^+$ ($Ly6G^+$) MDSC subsets in $CD11b^+$ cell population (percentages of gated populations) of Wild type (n=4) and $NLRP3^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**b, right**). Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by unpaired Student's t test. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

of inhibitor time-dependently, inhibited the release of IL-1β in splenocytes. Specifically, MCC950 markedly reduced the amount of ATP-induced NLRP3 mediated IL-1β secreted by the cells treated with it for 24 hours, confirming its strong inhibitory potency (Figure **20d**). However, splenocytes treated with the MCC950 inhibitor only for 2 hours showed no reduction in the secreted levels of IL-1β, compared with the cells that were not treated with it, and therefore no inhibition of NLRP3 inflammasome (Figure **20d**). Overall, these results determine the appropriate dose and the duration that MCC950 inhibitor requires to exert its inhibitory action on the treated cells, and thus to be used appropriately in our functional experiments.

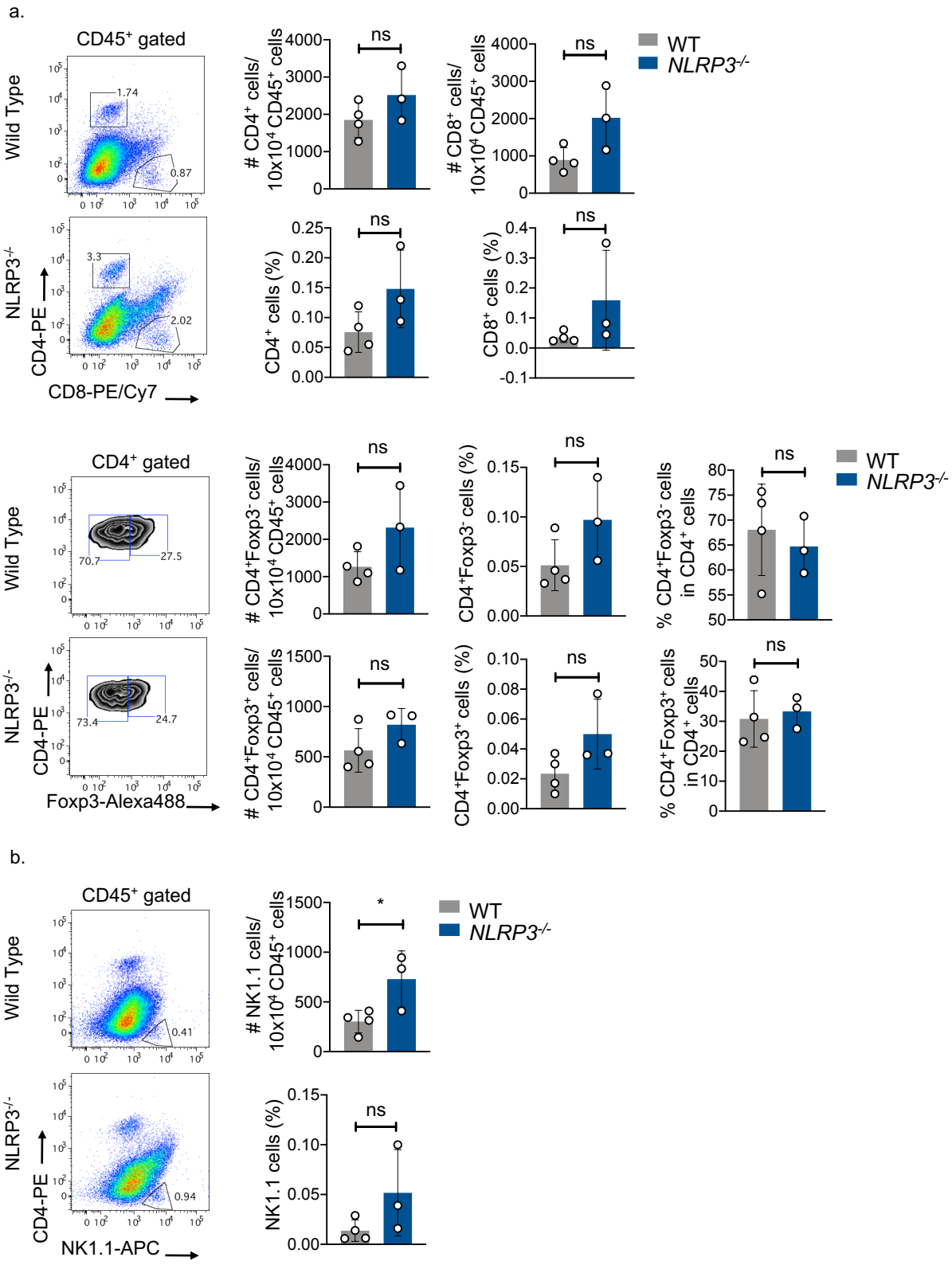

Figure 18. Effect of the NLRP3 inflammasome deficiency in tumor-infiltrated lymphoid cell compartment. (a) Gating strategy, numbers per 10×10⁴ CD45⁺ cells and frequencies in total tumor cells of CD4⁺, CD8⁺, CD4⁺Foxp3⁻ Teff and CD4⁺Foxp3⁺ Tregs cells in tumor sites of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation. For CD4⁺Foxp3⁻ Teff and CD4⁺Foxp3⁺ Tregs cells frequencies in CD4⁺ population are shown (percentages of gated populations). **(b)** Gating strategy, numbers per 10×10⁴ CD45⁺ cells and frequencies in total tumor cells of NK cells in tumor sites of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation. Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by unpaired Student's t test. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

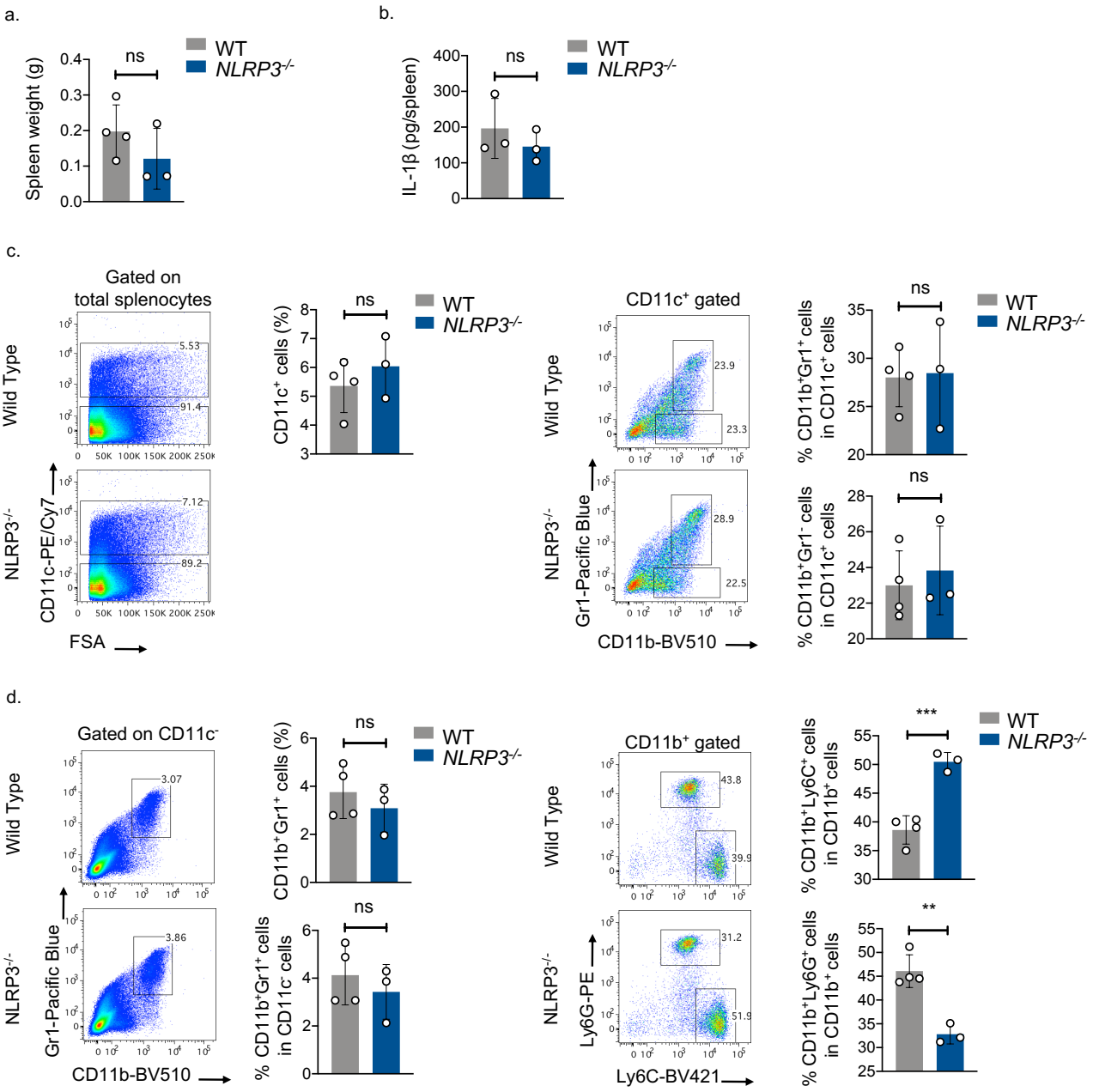

Figure 19. Effect of the NLRP3 inflammasome deficiency in spleen-infiltrated myeloid cell compartment. (a) Spleen weight of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice on day 19 after B16.F10 inoculation. **(b)** IL-1β levels (pg/spleen) in spleen homogenates from B16.F10-inoculated Wild type (n=3) and NLRP3$^{-/-}$ (n=3) mice on day 19. **(c)** Representative FACS plots, frequencies of spleen-infiltrating DCs (CD11c^{+}) in total splenocytes of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**a, left**); Representative FACS plots and frequencies of spleen-infiltrating CD11c^{+}CD11b^{+}Gr1^{+} (Gr1^{+}) and CD11c^{+}CD11b^{+}Gr1^{-} (Gr1^{-}) DC subsets in DC cell population (percentages of gated populations) of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**a, right**). **(d)** Representative FACS plots, frequencies of spleen-infiltrating MDSCs (CD11c^{-}CD11b^{+}Gr1^{+}) in total splenocytes and in CD11c^{-} cells (percentages of gated populations) of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**b, left**); Representative FACS plots and frequencies of spleen-infiltrating CD11b^{+}Ly6C^{high}Ly6G^{-} (Ly6C^{+}) and CD11b^{+}Ly6C^{low}Ly6G^{+} (Ly6G^{+}) MDSC subsets in CD11b^{+} cell population (percentages of gated populations) of Wild type (n=4) and NLRP3$^{-/-}$ (n=3) mice 19 days after B16.F10 inoculation (**b, right**). Data are shown as mean (±S.D.). Representative results from 3 independent experiments are shown. Statistical significance was obtained by unpaired Student's t test. Symbols: (ns), 0.1234; (*), p≤0.0332; (**), p≤0.0021; (***), p ≤0.0002; (****) p≤0.0001.

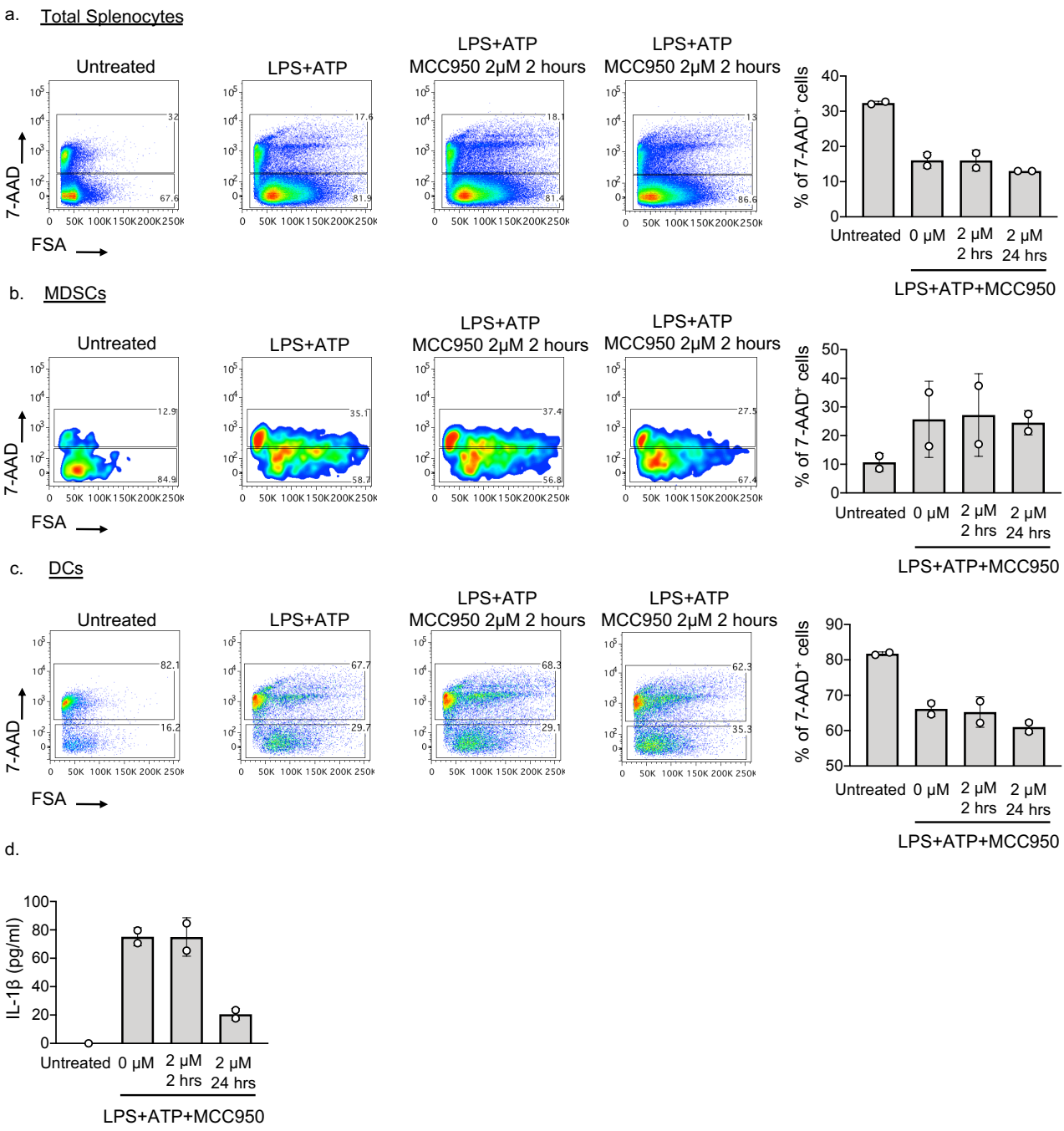

Figure 20. MCC950 inhibits NLRP3 inflammasome activation in response to LPS and ATP stimulation. Splenocytes isolated from two naïve mice (n=2, biologically independent mouse samples) were seeded into 96-well plates at density 5x10^5 cells/well. Splenocytes were primed for 24 hours using LPS (1μg/ml). Splenocytes were also treated with MCC950 NLRP3 inhibitor (2μM) for 2 and 24 hours, whereas control cells were treated with water for injection. One hour before the end of priming, cells were stimulated for NLRP3 inflammasome activity by ATP (5mM) inflammasome activator. One-hour post-NLRP3 inflammasome activation, stimulated splenocytes were collected, stained with 7-AAD viability staining solution and analyzed by flow cytometry for the estimation of the degree of apoptosis; and their supernatants were collected for IL-1β Elisa. **(a,b,c)** Representative FACS plots and bar graph plots showing the percentages of 7-AAD$^+$ cells for one of the different treatment conditions (n =2, biologically independent cell cultures) in total splenocytes **(a)**; in CD11c$^-$CD11b$^+$Gr1$^+$ MDSC gated population of splenocytes **(b)**; and in CD11c$^+$ DC gated population of splenocytes **(c)**. **(d)** IL-1β levels (pg ml^{-1}) in the supernatants of splenocytes stimulated with LPS and ATP and treated with MCC950 as measured by Elisa. Data are shown as mean (±S.D.). Representative results from 3 (**b**) and 1 (**a,c,d**) independent experiments are shown.

4. Discussion

A laborious effort in understanding tumor development and in the success of cancer immunotherapy is the determination of the role of soluble and cellular components that constitute the tumor microenvironment (TME) and enable it to subvert the host's immune system and suppress tumor immunosurveillance. Tumor's inflammatory microenvironment promotes an excessive and uncontrolled inflammation that damages the host immunity, which in turn fails to resolve itself, resulting in a chronic inflammatory state that has a huge contribution to tumorigenesis, with the underlying molecular mechanism still remaining unclear. Identification of cell-intrinsic pathways, which support cancer cells and favor malignant progression, could provide novel therapeutic strategies for tumor elimination. In the present Master Thesis, we provide evidence for the crucial role of the NLRP3 inflammasome pathway, a central signaling hub of the immune system, in the establishment of melanoma. Activation of the NLRP3 inflammasome pathway in myeloid cell compartment provided an inflammatory microenvironment promoting melanoma tumor progression. Moreover, our data suggest that inhibition of NLRP3 inflammasome attenuates melanoma tumor growth and alters the immune cell milieu.

TME is composed of heterogeneous mixture of cellular multitude which includes immune cell populations, from the lymphoid and myeloid lineage, and non-immune cell populations including tumor cells[13, 14, 15]. Depending on the context, infiltrating inflammatory cells in the TME may exert a dual role on tumor growth and progression. However, the soluble compartment of the TME, composed by protumoral factors and chemokines[16, 17, 18], regulate immune cells to favor tumor growth and progression. In solid tumors like melanoma, among tumor-infiltrating immune cells, a strong myeloid component has been reported, which has been accused as a critical regulator of tumor development and with a decisive role in therapeutic responses in cancer treatment[399]. Chronic inflammation in TME in tumor-bearing patients has been accused to induce the accumulation and retention of highly immunosuppressive myeloid cells, such as MDSCs[39]. In consistence with this, the highly inflammatory and immunosuppressive TME promotes tumor growth and development through the aberrant activation of myelopoiesis resulting in the expansion and recruitment of immature myeloid cells[39, 40]. Our results confirm this strong myeloid signature during melanoma tumor development and progression. Specifically, the highly immunosuppressive MDSC population despite the fact that during the different days of tumor development showed no alterations in its frequencies in tumor tissue, in peripheral lymphoid organs such as spleen demonstrated a gradual increase. Additionally, we observed that the proportion of PMN-MDSCs in the spleen was much higher than that of M-MDSCs, whereas in tumor tissue M-MDSCs account for a greater proportion. All the above pointed toward a strong suppressive function of MDSCs in tumor tissue, as M-MDSCs are reported to be a more suppressive MDSC subset population than PMN-MDSCs, providing thus a suitable immunosuppressive microenvironment for tumor development and progression. In spite of the moderate suppressive activity of PMN-MDSCs, have been reported to play a major role in the regulation of tumor-specific immune responses, ultimately leading to the development of tumor-specific T cell tolerance, underlying thus the crucial role of their high proportion in peripheral lymphoid organs. Regarding the DC population, our results demonstrated no differences in the frequencies of tumor-infiltrating DCs between the different stages of tumor development, whereas in spleen tissue a notable decrease was observed at the eighth day of tumor development, reaching at the end point the same numbers as at the naïve state. Observing and interpreting our results, we expect that as the tumor grows, the proportion of MDSCs that infiltrate the tumor will be increased compared to the DC population, which is known to mediate both priming and tolerizing antitumor T-cells in the TME[98, 408], providing thus an immunosuppressive microenvironment for tumor development. However, our results demonstrate that during tumor progression both in spleen and tumor tissue the frequencies of DCs were notably elevated compared of those of MDSCs. A possible explanation can be found in the proportion of $Gr1^-$ and $Gr1^+$ subsets in the DC population, as the $Gr1^+$ DC subset population is called inflammatory DCs, appear during inflammation and derive from monocytes that differentiate in situ at the site of inflammation, endocytose tumor antigens and transport them to the draining lymph nodes where they prime antigen-specific $CD4^+$ and $CD8^+$ T cells[109, 126], whereas the $Gr1^-$ DC subset population mediate T-cell anergy and tolerance by presenting antigen in the context of decreased surface expression of costimulatory molecules and production of the anti-inflammatory cytokines[106, 409]. In support of this, DCs were represented by $Gr1^-$ cell subset in spleen, in contrast to TME where the $Gr1^+$ cells showed an increased proportion.

Chronic inflammation is recognized as an important event in carcinogenesis and tumor progression. Inflammasomes and their effectors, such as IL-1β, are able to shape the tumor milieu through their contribution to inflammation and immune responses, affecting thus the development, progression and treatment of cancer, which depicts the diverse roles of inflammasomes in modulating carcinogenesis and their potential targeting in

translational research. Supporting evidences documented overactivation of the IL-1β system across many cancer types, including fibrosarcoma, lung cancer, pancreatic cancer, melanoma, breast cancer and colorectal cancer[322, 323, 324, 325]. In addition, alteration of NLRP3 expression and activity combined with high levels of IL-1β in plasma and tissues, are bad prognostic biomarkers in experimental tumor models and in cancer patients and associated with carcinogenesis and tumor invasiveness[189, 326, 327, 328]. Although the well-documented clinical and experimental evidence of pro-tumorigenic role of IL-1β-NLRP3 inflammasome axis, its role in cancer remains controversial. To shed light in this controversial issue we determined IL-1β levels in spleen and tumor tissues of our B16-F10 melanoma models, at different days after the tumor induction with Elisa. Our data demonstrate that the presence of IL-1β in tumor site and spleen tissue of melanoma-bearing mice is associated with tumor progression and development, suggesting a pro-tumorigenic role for IL-1β.

Depending on the preclinical models used, the role of the inflammasome in carcinogenesis has been contradictory and context dependent. The controversial role of NLRP3 inflammasome signaling in promoting tumor growth or enhancing tumor-suppressive effects might be correlated to the cell lineage involved. The relative expression of inflammasome components differs in various cell types, which suggests that inflammasomes perform distinct functions in different cellular compartments. A study by the team of Jürg Tschopp in 2011[160], documented the elevated expression of NLRP3 upon exposure to inflammatory stimuli primarily by myeloid cells, such as splenic neutrophils, macrophages, monocytes and conventional dendritic cells, whereas NLRP3 was barely expressed by lymphoid subsets (splenic lymphoid B, T, and NK cells), eosinophils and plasmacytoid dendritic cells. Also, several studies reported that IL-1β is mainly expressed in myeloid cells[209, 210, 283]. Activation of the NLRP3 inflammasome in MDSCs was previously shown in a study by Van Deventer et al. in which the expression of Nlrp3 in the tumor microenvironment diminishes antitumor immunity and dendritic cell vaccination efficacy by facilitating the migration of MDSCs to the tumor site in a melanoma model[176] and also in study by Bruchard et al. suggesting that chemotherapy-mediated activation of NLRP3 in MDSCs limits its antitumor efficacy and is a positive regulator of cancer growth[43]. However, the expression of NLRP3 inflammasome in myeloid compartment, specifically in MDSCs, and inflammasome's functional role in tumor growth has not been thoroughly investigated. Taking the above into consideration and in combination with the strong myeloid signature during melanoma tumor development and progression we decided to examine the expression of NRLP3 inflammasome signaling in DC and MDSC myeloid cell types in tumor and spleen tissues during tumor progression. We found that both in naïve and tumor state, DC and MDSC populations substantially express NLRP3 and pro-IL-1β, thus answering the question whether these myeloid populations we have chosen to study express the NLRP3 inflammasome pathway upon exposure to stimuli, and in this case in tumor condition. Expression of NLRP3 inflammasome pathway in DCs and MDSCs not only in later but also in earlier stages of tumor development, combined with the presence of IL1-β in tumor and spleen tissues during tumor development and the knowledge that NLRP3 and IL-1β mainly expressed in myeloid cells[160, 209, 210, 283], motivated us to answer the question if these two myeloid cell populations express NLRP3 and pro-IL-1β more than any other of the cell population that infiltrate tumor and spleen tissues, and if so which of the two has greater expression and consequently stronger activation of the NLRP3 inflammasome signaling. Our results demonstrate that tumor- and spleen-infiltrating DC and MDSC myeloid populations showed elevated levels of NLRP3 and pro-IL-1β during tumor development, compared with the rest $CD45^+$ leukocytes which are not DCs and MDSCs and infiltrate tumor and spleen tissues. The comparison of these two myeloid populations pointed out tumor- and spleen-infiltrating DCs as the myeloid population with the highest levels of NLRP3 expression. Paradoxically, these upregulated levels of NLRP3 were not always accompanied by the same increase in pro-IL-1β expression in DCs, as only in tumor tissue DCs reported increased or same pro-IL-1β levels compared with MDSCs, whereas in spleen, despite the elevated expression of NLRP3 in DCs, MDSCs showed increased production of pro-IL-1β. Interpreting our results, the increased levels of NLRP3 protein in DCs can be attributed to the fact that it belongs to one of the six classes of PRRs, which are mainly expressed in antigen-presenting cells (APCs) at the front line of defense, such as dendritic cells (DCs), monocytes and macrophages[147]. Activation of the NLRP3 inflammasome in these cells, such DCs, has also been reported to induce IL-1β-dependent adaptive immunity against tumors[167]. However, this does not change the fact that NLRP3 inflammasome is expressed in MDSCs, and even at high levels, and that despite its increased expression in DCs, MDSCs appear to contribute more to the production of IL-1β.

Moreover, our findings on the determination of NLRP3 and pro-IL-1β levels in the different subsets of DCs and MDSCs myeloid cells demonstrated increased NLRP3 expression in tumor-infiltrating M-MDSCs ($Ly6C^+$) at the endpoint of tumor progression, whereas in spleen-infiltrating MDSCs elevated NLRP3 signaling reported on PMN-MDSCs ($Ly6G^+$) subset suggesting that the NLRP3 inflammasome maybe induce more the suppressive nature of MDSCs. One could hypothesize that the increased proportion of M-MDSC suppressive subset in tumor together

with its increased NLRP3 expression maybe indicates a suppressive function for NLRP3 inflammasome. Similar, increased proportion of PMN-MDSCs in spleen accompanied by elevated NLRP3 signaling maybe suggests a possible involvement of NLRP3 inflammasome in regulation of tumor-specific immune responses. Regarding the DC subsets, our data revealed increased NLRP3 and pro-IL-1β expression in $Gr1^+$ inflammatory DCs that infiltrate spleen tissue in naïve and tumor state, despite the fact that DCs are mostly represented by the $Gr1^-$ cell subset in spleen, whereas in tumor tissue the increased proportion of $Gr1^+$ cells is accompanied by their increased NLRP3 signaling, suggesting thus a possible role in their function in regulating immune responses.

The regulation, the processing of IL-1β from its inactive pro-form and the bioavailability of the mature bioactive IL-1β cytokine is a complex and controlled process. The NLRP3 inflammasome is currently the most thoroughly characterized inflammasome that controls IL-1β release in the innate immune system[148, 294]. The main characteristic of IL-1β protein is that it takes an unconventional secretory pathway to exit the cell and the reason is the lack of the signal peptide[306], indicating that IL-1β secretion is independent of conventional secretion pathway, which includes the ER together with the Golgi apparatus, and follows a non-conventional secretion route[307]. Our in vitro experiments confirmed this knowledge, demonstrating that IL-1β secretion is mediated independently of ER/Golgi trafficking route in MDSCs and DCs myeloid cells, so there is no need for treatment of cultured cells with Brefeldin A, a fungal metabolite that inhibit the protein transport through the collapse of Golgi apparatus leading to the accumulation of most cytokines at the ER/Golgi trafficking route, for the determination of intracellular levels of pro-IL1-β. In addition, we demonstrated that MDSCs and DCs constitutively express NLRP3 and pro-IL-1β, and the expression of these genes was up-regulated by stimulation with LPS, suggesting that the NLRP3 inflammasome expressed in these myeloid populations may play an important role in immune responses. We also found that ATP treatment alone not only failed to induce NLRP3 and pro-IL-1β expression in cultured splenocytes and MDSCs, but also failed to induce IL-1β secretion in cultured MDSCs. This may be because without LPS treatment, the expression level of pro-IL-1β is minimal in both MDSCs and DCs. IL-1β secretion induced by ATP was detected in LPS-primed MDSCs, indicating that MDSCs are able to secret IL-1β upon cellular infection, thus contributing to innate immune responses in TME, leading to chronic inflammatory states. Moreover, we analyzed the effect of tumor explant supernatant (TES) from B16.F10 induced melanoma tumors, as it contains multiple DAMPs derived from necrotic cancer cells. Importantly, expression analysis showed that TES was not a potent inducer of the NLRP3 inflammasome in MDSCs and DCs, as both the NRLP3 and pro-IL-1β expression was not upregulated by the TES treatment alone or by ATP and TES, with the inflammasome signaling to be induced in MDSCs only in presence of LPS stimulus.

Currently, the role of inflammasomes in tumor initiation and growth remains not fully understood. As already mentioned, the expression of IL-1β is elevated in a variety of cancers, including fibrosarcoma, lung cancer, pancreatic cancer, melanoma, breast cancer and colorectal cancer[322, 323, 324, 325]. The pro-tumorigenic effects of the IL-1β-NLRP3 inflammasome axis are associated with the ability of IL-1β to act as a mediator of chronic non-resolving inflammation[321], tumor angiogenesis, endothelial cell activation and recruitment of immunosuppressive cells and thus resulting in immunosuppression, tumor development and progression, and metastasis[321]. Our data suggest that NLRP3 inhibition has protective effects in B16.F10-melama bearing mice, as attenuated tumor growth was reported in mice deficient for NLRP3 inflammasome, compared with the control animals. In support of our findings that NLRP3 signaling promotes melanoma progression, many studies have revealed that NLRP3 inflammasome and IL-1β contribute to tumor development. Van Deventer et al. investigated the role of the NLRP3 inflammasome in the immune response induced by a dendritic cell vaccine against the poorly immunogenic melanoma cell line B16-F10. The authors suggested that the expression of NLRP3 in the tumor microenvironment diminishes antitumor immunity and vaccine efficacy by facilitating the migration of MDSCs to the tumor site, thus proposing another deleterious mechanism of action of the NLRP3 inflammasome in the context of cancer[176]. Moreover, Daley et al. showed that NLRP3 signaling promotes accelerated progression of pancreatic neoplasia and suggested it as a novel target for reprogramming the TME toward an immunogenic innate and adaptive inflammatory phenotype[162]. In addition, another study by Bruchard et al. demonstrated genetic inactivation of NLRP3 enhanced the antitumor efficacy of 5-fluorouracil chemotherapeutic agent against EL4 thymoma growing. However, NLRP3 deficient mice without 5-fluorouracil treatment showed no reduction at tumor growth[43].

Both our results and results that support the pro-tumorigenic role of NLRP3 inflammasome contrasts with studies indicate that inflammasomes generally provide protection against tumorigenesis with the most of them to being demonstrated in gastrointestinal cancers. For example, Ghiringhelli and colleagues[167] showed that the NLRP3 inflammasome was critically important in the P2RX7-dependent activation of DCs to generate IFN-γ-producing $CD8^+$ T cells. In addition, in colon cancers, the genetic absence of NLRP3 inflammasome components, such as NLRP3, ASC

and caspase-1 proteins, increased the susceptibility and morbidity to cancer inflammasome and is associated with more aggressive tumor growth[173, 174, 376, 386, 387]. As we understand the role of NLRP3 inflammasome in cancer remains controversial. Although our findings in melanoma context are seemingly contrary with some studies, the patterns are not necessarily paradoxical, as the effects of NLRP3 activation in tumor progression may be contingent not only on the differential roles played by immunity and inflammation in each specific malignancy, but also on the functional role of inflammasome of the cell lineage which is involved, determining thus the propensity for tumorigenesis.

Importantly, we assessed the IL-1β levels in tumors and spleens of NLRP3$^{-/-}$ and WT tumor-bearing animals and paradoxically no difference was found between the two groups. In contrast, a study from Guo et al. found reduced levels of IL-1β in tumor tissues from NLRP3 and caspase-1 deficient mice injected with EO771 tumor cells[334]. Despite these contradicting results, a possible explanation for our findings can be supported by a recent study of Theivanthiran et al.[261] reported the activation of NLRP3 inflammasome in cancer cells, and thus the increased IL-1β levels in tumor tissue of our NLRP3$^{-/-}$ mice maybe are derived from melanoma cells, which they have a fully functional inflammasome. Regarding the elevated levels of IL-1β in the spleen of NLRP3$^{-/-}$ mice, one could suggest that IL-1β is secreted in large amounts by cancer cells and through the systemic circulation reaches the spleen, raising its levels there as well. Alternatively, one could hypothesize that the IL-1β which is detected in spleen tissue comes from the spleen-infiltrating cells but is produced through an NLRP3-inflammasome independent pathway[410].

Analysis of immune cell infiltration in tumors of WT and NLRP3$^{-/-}$ mice indicated that attenuated tumor growth in NLRP3 deficient mice, was associated with no difference in the infiltration of CD45^{+} leukocytes, increased proportion of CD11c^{+}CD11b^{+}Gr1^{+} (Gr1^{+}) DC cell subset and markedly decreased frequencies of CD11c^{+}CD11b^{+}Gr1^{-} (Gr1^{-}) cell subset, whereas no alterations in the frequencies of DC and MDSC populations were noted, regarding the myeloid compartment. The changes in recruitment of the PMN-MDSCs (CD11b^{+}Ly6C^{low}Ly6G^{+}) and M-MDSCs (CD11b^{+}Ly6C^{high}Ly6G^{-}) subpopulations were distinct in the spleen of NLRP3$^{-/-}$ tumor-bearing mice, where MDSCs were highly represented by M-MDSCs subpopulations. In the lymphoid cell compartment, paradoxically tumor regression in NLRP3$^{-/-}$ mice was not accompanied by increased frequencies of CD4^{+} and CD8^{+} T cells, suggesting a robust antitumor immune response, but it was associated with increased recruitment of NK1.1^{+} cells. NK cell responses play an important role in antitumor immunity by exerting strong cytotoxic activity, whereas inflammatory DCs (CD11c^{+}CD11b^{+}Gr1^{+}) endocytose tumor antigens and transport them to the draining lymph nodes where they prime antigen-specific CD4^{+} and CD8^{+} T cells[109, 126]. Thus, our findings suggest that an anti-tumor immune response based on these two populations, combined with the decreased infiltration CD11c^{+}CD11b^{+}Gr1^{-} DC subpopulation in TME, which mediate T-cell anergy and tolerance by presenting antigen in the context of decreased surface expression of costimulatory molecules and production of the anti-inflammatory cytokines[106, 409], is responsible for tumor regression observed in the mice with NLRP3 deficiency. In addition, recruitment of PMN-MDSCs into tumor tissues have been linked with a tumor-intrinsic PDL-1-NLRP3 inflammasome signaling pathway, which drives resistance to anti-PD-1 immunotherapy and dampen the resulting anti-tumor immune response[261]. This finding is in-line with a previous study showing that NLRP3 can mitigate against DC vaccine therapies by promoting the migration of MDSCs into tumors[176]. These studies together with our findings about the reduced recruitment of the PMN-MDSCs subpopulation in the spleen, reveal that NLRP3 inhibition suppress the recruitment of PMN-MDSCs and maybe has an effect in induction of antitumor immunity.

Last but not least, we used the MCC950 inhibitor to inhibit the NLRP3 inflammasome in vitro. MCC950 agent that has the ability to specifically inhibit the activation of both the canonical and the noncanonical NLRP3 inflammasomes, while it does not affect and impairs the activation of AIM2, NLRC4, or NLRP1 inflammasome[238, 239, 240]. Our data demonstrate the inhibitory action of MCC950 inhibitor through the reduction of secreted levels of IL-1β in vitro, thus highlighting its use as a useful tool for exploring NLRP3 druggability in mice in vivo.

Collectively, the inflammasome signaling is closely associated with many human cancers and exhibits conflicting roles in multiple aspects of tumorigenesis. Our findings delineate a pivotal role of NRLP3 inflammasome in the establishment of melanoma. With our study, new insights on inflammasome signaling in human cancers are offered. Undoubtedly, a deeper understanding of the NLRP3 inflammasome activation in the context of melanoma will result in a better mechanistic insight of how it influences tumor progression and anti-tumor immune responses. Also, findings will shed light on the involvement of the NLRP3 inflammasome of specific tumor-infiltrating cell subtypes in the establishment of cancer. Continuation of this project is expected to open new avenues for translational research, which will undoubtedly lead to development of more efficacious immunotherapeutic approaches.

5. Future Directions

Nowadays it has become clear that when it comes to cancer, there is no silver bullet. No single solution solves all problems and for this reason the future of cancer care is a combination therapy, which will combine immunotherapy with therapies that improve the function of anti-tumor immunity and target the function of cells that suppress it. Thus, for our future experiments we aim to investigate the effect of a combination therapy, which will combine the immunotherapy with the specific inhibition of the NLRP3 inflammasome, in tumor growth and tumor immune response. For this reason, we will use our NLRP3 deficient mouse model to examine the immunotherapeutic effects, and to translate these findings in clinical practice we will assess the efficacy of chemopreventive inhibition of NLRP3 inflammasome by the specific small molecule inhibitor MCC950, which will pave the way for a novel therapeutic strategy in cancer treatment.

Further on, through flow cytometric analysis and suppression assays, we are going to determine how the NLRP3 inflammasome deficiency affects the suppressive function of MDSCs. Specifically, through flow cytometric analysis we aim to examine the expression of specific suppression markers of MDSCs that infiltrate tumor microenvironment and peripheral lymphoid organs, such as spleen, of wild type and NLRP3 deficient mice. Also, we intend to examine the functional properties of NLRP3 inflammasome deficient MDSCs. Thus, we will sort highly pure MDSCs from spleens of $NLRP3^{-/-}$ and control mice and examined their suppressive properties in vitro. Furthermore, coinjection of MDSCs from tumor-inoculated $NLRP3^{-/-}$ mice with B16-F10 cells or adoptive transfer of NLRP3 inflammasome deficient MDSCs into tumor-bearing wild type mice or vice versa, will also determine the suppressive activity of NLRP3 inflammasome deficient MDSCs in vivo.

Moreover, to exclude immune cell alterations in $NLRP3^{-/-}$ mice at steady state, naive $NLRP3^{-/-}$ mice will be compared with naïve WT animals. Therefore, we will assess the immune cell infiltration in peripheral lymphoid organs of naïve mice, so to be sure that any difference in their immune response to melanoma is solely due to the way that the NLRP3 inflammasome deficiency shapes the anti-tumor immune response and not to immune cell alterations at steady state.

Last but not least, in order to elucidate the molecular mechanism through which NLRP3 inflammasome maybe affects the recruitment and the suppressive function of the PMN-MDSCs and M-MDSCs subpopulations, we intend to perform transcriptomic analysis (RNA-seq) of PMN-MDSCs and M-MDSCs isolated from tumor-bearing $NLRP3^{-/-}$ and control animals. Thus, PMN-MDSCs and M-MDSCs will be sorted from the spleens of B16.F10 melanoma cell-inoculated $NLRP3^{-/-}$ and wild type mice for identification of differentially expressed genes, which will yield valuable insight into the NLRP3 inflammasome signaling of cancer MDSCs.

All these experiments will provide us evidence for a deeper understanding of inflammasome's biology in cancer and for the effective design of novel therapeutic protocols in cancer.

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