To my brother

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Role of the inflammasome in lung cancer
Inflammasome is involved in lung carcinogenesis

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ABSTRACT

Lung cancer is recognized as one of the most devastating tumor worldwide due to the low rate survival over 5 years from the time of diagnosis. Inflammation has been widely recognized as the seventh hallmark of cancer as it facilitates the establishment/development and progression of lung cancer. In this context, recent evidence highlighted the role of the inflammasome during carcinogenesis. However, little is still known. The inflammasome is a multiprotein complex that leads to caspase-1 activation which role in lung cancer is still under investigated. In this context, the aim of my PhD project was to understand the role of the inflammasome in lung cancer in a mouse model of carcinogen-induced lung cancer and in human non-small cell lung cancer (NSCLC). We found that both caspase-1-dependent, the canonical pathway, and caspase-8/caspase-11-dependent, the non-canonical pathway, inflammasome were involved during lung cancer establishment and progression in both mice and humans. Our data showed that the pharmacological inhibition of both caspase-1 and caspase-8 significantly reduced lung tumor outgrowth associated to lower pro-inflammatory response and to a reduced lung recruitment of immunesuppressive cells and that caspase-8 was upstream caspase-1 activation during lung carcinogenesis. Furthermore, we showed that caspase-11 was the primary/main orchestrator of the inflammasome-dependent lung cancer progression and that the enzyme could be upstream of caspase-1 to induce the amplification of the occurring inflammatory process associated to lung cancer development. Finally, we identified a novel mechanism by which lung tumor-associated macrophages could favor lung tumorigenesis via the activation of caspase-11-dependent inflammasome and the consequent release of the pro-tumorigenic IL-1α.

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INTRODUCTION

Lung cancer is a devastating disease that counts more deaths than other solid tumors, with a 5-year survival rate of 10%[1]. The number of new cases is expected to rise by about 70% over the next two decades. More dramatically, over half of people with lung cancer die within one year of being clinically diagnosed due to a still unmet medical need: 1. lack of prevention and early detection tools and 2. recognition of the symptoms at the sole late stages[1]. ***To note, the sole computed tomography (CT) scan and surgical resection accompanied by chemo- and radiotherapy are actually the options for lung cancer patients.***

The actual chemotherapy for lung cancer patients includes inhibitors of cell proliferation (i.e. carboplatin, docetaxel, gemcitabine). Besides their side effects, the efficacy of these drugs is very low and more importantly they are preferred after surgical resection. Other innovative drugs are represented by anti-angiogenesis therapy (antibodies against vascular endothelial growth factor, VEGF) and by epidermal growth factor receptor (EGFR) blockade (erlotinib), anaplastic lymphoma kinase (ALK) inhibitors (crizotinib, ceritinib, and alectinib) and the recent discovered anti-PD-1 antibodies (nivolumab and pembrolizumab). While anti-PD-1 antibodies proved efficacy in melanoma patients, they are still an imperfect therapeutic tool for lung cancer patients, who still have a low survival rate even after the association with anti-CTLA4 antibody (ipilimumab)[2]. Therefore, two major issues raise: 1. the classical chemotherapy is not as efficient as the proliferation rate of neoplastic cells, which overcomes the pharmacological efficacy in favour of high risk side effects; 2. the percentage of patients that can be treated with the above innovative drugs (i.e. nivolumab) are around 20-30% with a relative survival rate of 3 months compared to patients solely treated with the conventional

therapy[3]. ***Therefore, it is obvious that there is a clinical unmet need for lung cancer patients.***

In order to attempt a novel therapeutic approach for lung cancer patients, we started from a hallmark of lung cancer, chronic inflammation. Several lines of studies evidence that chronic inflammation underlies lung carcinogenesis. The proposed role of chronic inflammation in the tumorigenic process is based on a 2-stage model of carcinogenesis[4], during which tumor formation involves an initiating event, such as a genotoxic injury, followed by promotional events and/or conditions that result in clonal growth of transformed cells. In this context, chronic inflammatory responses have been proposed as an endogenous (sterile) source of mutational events: the altered frequency of cell proliferation and of metaplasia in epithelial tissues can contribute to regenerative events that while they intend to replace the damaged epithelium, they turn into enhancing the probability of somatic mutations, especially in an inflammatory microenvironment. In addition, in response to a variety of inflammatory cytokines genotoxic metabolites by neighbouring epithelial and stromal cells can also be produced. Moreover, many tumors are associated with the infiltration of inflammatory cells that in most cases, due to their immune-suppressive nature, are related to a bad prognosis[5,6]. Chronic inflammation allows malignant cells to escape from or suppress anti-tumour immunosurveillance mechanisms[5,7–9].

Therefore, the prolonged contact of respiratory epithelial cells and the lining immune cells with insulting molecules can initiate and sustain inflammatory responses. Epidemiological studies revealed that lung chronic inflammation initiate/promote the development of lung malignancy, possibly in conjunction with tobacco use and/or other environmental pollutants (i.e. asbestos, silica, diesel exhaust)[4]. Indeed, elevated serum levels of C-reactive protein (CRP) are associated, together a particular lifestyle (i.e. smoking, air pollutant exposure) to increased risk of lung cancer[10,11]. Concomitantly, high levels of the pro-

inflammatory cytokines, such as IL-1β and IL-18, are detected in the plasma and tissue of lung cancer patients, identified as bad prognostic biomarkers[12]. IL-1-like cytokines (i.e. IL-1α, IL-1β, IL-18 and IL-33) are identified as 'alarmins'. Their expression is tightly regulated by multiprotein complexes referred to as 'inflammasomes'[13].

The inflammasome is a multicomplex system that comprises the activity of several proteins that promote caspase-1 activation (Figure 1)[13]. Endosomal and extracellular Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors (NLRs) are a class of sentinel receptors that are pivotal in the detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Figure 1)[13,14]. The cooperation between these two systems allows to 'sense', and respond to a large number of infectious and sterile insults. Initially, NLRs were proposed to regulate inflammation through apoptosis, but nowadays this concept has been modified in that NLRs may serve as sentinels for cellular distress[14,15]. Several NLRs species have so far been identified in both humans and mice, *i.e.*, NLRP1, NLRP3, NLRP6, NLRC4, AIM2[15]. These proteins recognize distinct signals and, most importantly, are expressed at different levels. Full activity of NLRP3, the most studied NLR to date, requires two signals: the first induces its expression, along with synthesis of pro-IL-1β, via NF-κB activation, and the second activates NLRP3 that assembles to the adapter protein apoptosis associated speck-like protein containing a CARD domain (ASC) for the activation of caspase-1 (Figure 1)[13,15]. In contrast, NLRC4 and AIM2 are constitutively expressed in hematopoietic cells and are triggered by flagellin-like molecules and dsDNA, respectively[15]. The common activity to all NLRs is the activation of caspase-1 which converts pro-IL-1β and pro-IL-18 into their active isoforms (Figure 1)[13].

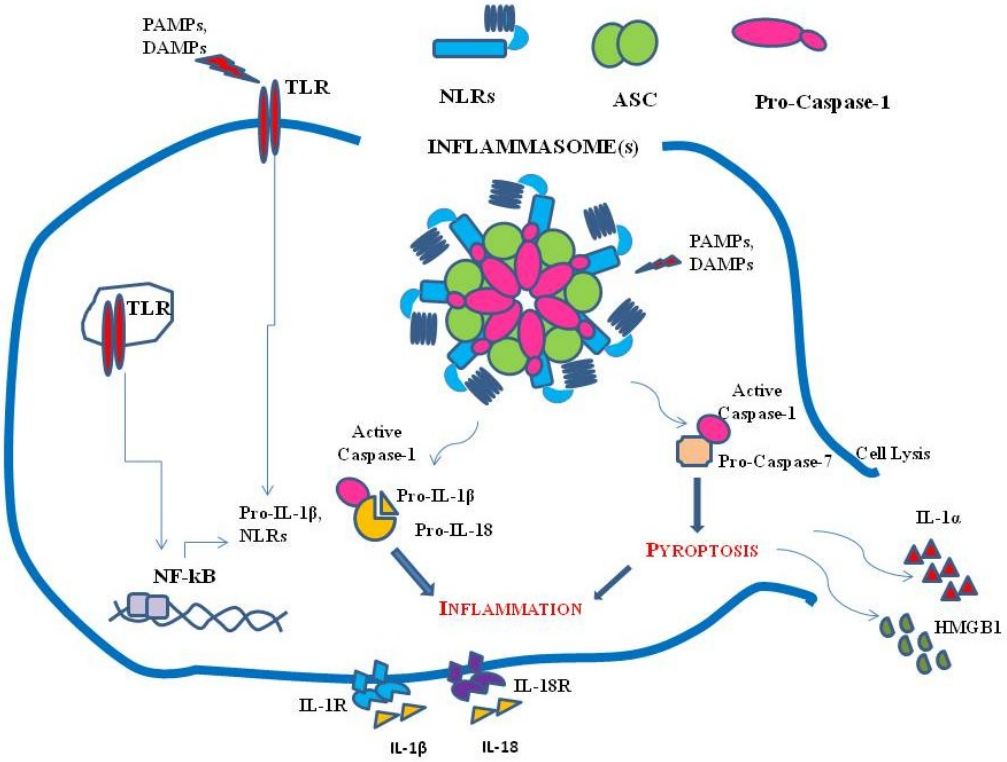

Figure 1. The inflammasome is a multicomplex system which activation is induced either directly by specific PAMPs and/or DAMPs or by the two-signal model as in the case of NLRP3. The recognition of PAMPs and/or DAMPs by extracellular or cytoplasmic TLRs leads to the activation of NF-κB (first signal), which in turn promotes the transcription of pro-IL-1β/IL-18 or some NLRs (e.g. NLRP3). NLRs assemble into the inflammasome complex which via the CARD domain can recruit pro-caspase-1 and promote its autocatalytic cleavage (second signal). Caspase-1 can lead to a cascade of pro-inflammatory events via the activation of pro-IL-1β and pro-IL-18, which then interact with their own membrane receptors amplifying the inflammatory response. On the other hand, active caspase-1 can lead to cell pyroptosis with the consequence of membrane rupture and release of such alarmins as IL-1α and high mobility group box 1 (HMGB1)[13].

The third effector mechanism of the inflammasome activation, besides the release of the active IL-1β and IL-18, is the induction of pyroptosis, a cell death process that requires the activity of caspase-1 and a critical mechanism by which inflammasomes contribute to host responses against Gram-negative and Gram-positive bacteria[15]. Pyroptotic cell death has been well described in the hematopoietic cell lineage but it can also occur in stromal cells such as in the central nervous system and cardiovascular system in ischemic and autoimmune conditions[16].

Alternative, non-canonical inflammasomes have also been described, which engage caspase-11 (also known as caspase-4/5 in humans). This in turn induces inflammasome-dependent caspase-1 activation and inflammasome-independent, pyroptosis-like cell death, via the release of such 'alarmins' as IL-1α and high mobility group box 1 (HMGB1)[17].

Because IL-1α/β, IL-18 and pyroptosis have the potential to damage the host, tight control of these pathways is critical for the prevention of chronic inflammation. These processes are key steps for the regulation of programmed cell death, differentiation and proliferation[16], three aspects that in the context of cancer represent the rheostat for tumor proliferation versus tumor arrest/regression (Figure 2).

In the face of an ever-expanding bulk of literature on the role of inflammasome in host defense, little is known about its role in lung cancer. So far, in the context of tumor microenvironment, the activation of the inflammasome has yielded to conflicting results and interpretation.

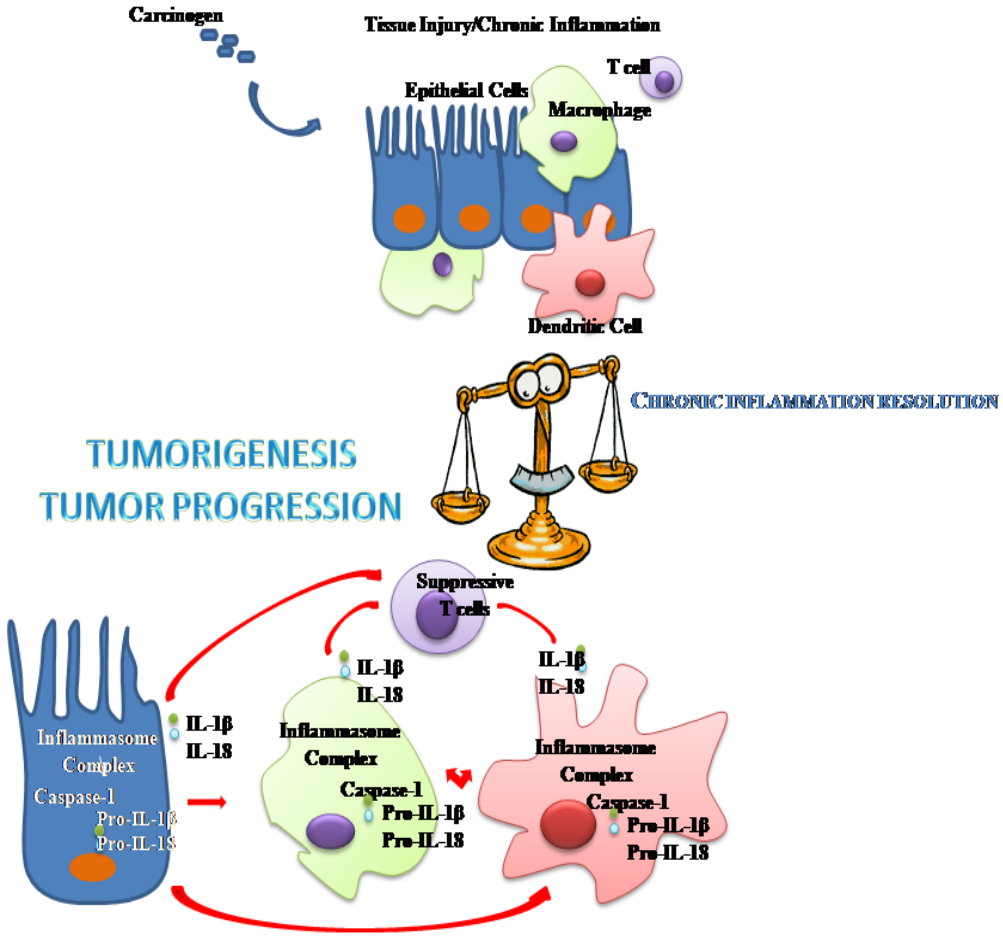

Figure 2. Chronic inflammation is at the interface between cells injury resolution and tumorigenesis. The activation of the inflammasome in epithelial cells, dendritic cells and macrophages maybe responsible for chronic inflammation at the basis of lung carcinogenesis. Therefore, the inflammasome complex may represent the rheostat for tumor establishment/progression versus tumor arrest/regression.

Pro-tumor activity of the inflammasome. Epidemiological studies showed that high serum concentrations of pro-inflammatory inflammasome-related cytokines, e.g. IL-1β/IL-18, are correlated to malignancies with low-rate survival from time of diagnosis[18]. These cytokines, together with TNF-α and IL-6, which can be induced by IL-1-like cytokines[19], are involved in cell proliferation and survival[20], as well as cell adhesion and migration[21,22], all features of tumor progression and invasiveness.

In the tumor microenvironment, IL-1-like cytokines can be secreted by both malignant and infiltrated immune cells[13,23] (Figure 3)[13]. Tumorigenesis, tumor progression and dissemination, and tumor immune-editing are affected by the presence or not of these cytokines which can mediate a variety of local and systemic activities.

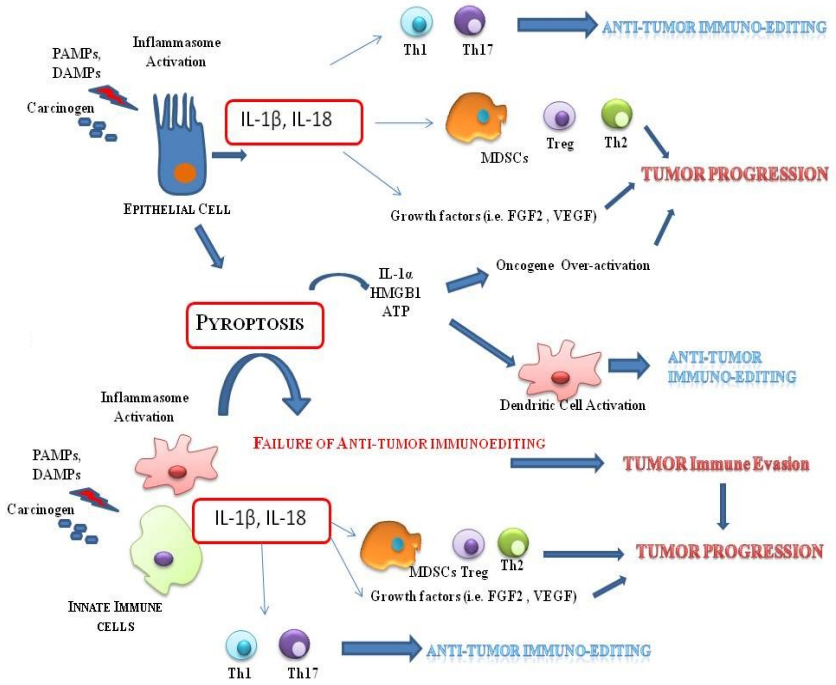

Figure 3. The exposure to carcinogens or intrinsic genetic aberrations are at the basis of cancer initiation. The activation of the inflammasome in epithelial cells, dendritic cells and macrophages may lead to different immune responses according to the tumor microenvironment[13].

IL-1α is an alarmin that under physiological conditions is present into the cell in its precursor form[24]. Upon inflammatory conditions, it is cleaved by the protease calpain[25] and/or caspase-11[26], with the ensuing inflammation/necrosis and subsequent adaptive immune responses[27]. In a model of diethylnitrosamine (DEN)-induced liver carcinoma[28], skin papillomas[29] and gastric carcinoma[30], IL-1α is released by dying cells, stimulating oxidative stress pathways responsible of local inflammation and in some cases of cell rescue from death to provide tissue regeneration and subsequent accumulation of mutations and tumor initiation/progression[31]. In support of these findings, IL-1α-induced IL-6 activates STAT-3 and promotes liver as well as gastric tumorigenesis[28–30]. An indirect evidence for IL-1α-dependent tumor outgrowth is provided in studies of IL-1R1- and MyD88-deficient mice, which develop less skin[32], colon[33] and liver[34] tumor lesions. Besides its role in tumor-associated inflammation, IL-1α activity was also associated to the activity of mutated K-Ras, one of the main oncogenes, which in turn induced constitutive activation of NF-κB and AP-1 that on one side promoted an autocrine loop for further IL-1α expression/secretion, and on the other increased tumor burden in a mouse model of pancreatic carcinoma[35]. In sharp contrast, in a mouse model of methylcholanthrene (MCA)-induced fibrosarcoma IL-1α knockout mice had similar tumor lesions as wild type mice[31]. This implied that it was not implicated in tumor outgrowth, but rather, cell-membrane exposed IL-1α exerted anti-tumor activity in that it edited tumor immune-surveillance via the activation of NK, $CD4^+$ and $CD8^+$ T cells. Some cancer cells can express membrane IL-1α, which can increase the immunogenicity of tumor cells and promote anti-tumor immune surveillance and tumor regression. However, high levels of IL-1α in the tumor microenvironment can favor angiogenesis and invasiveness[27].

Similarly, IL-1β and IL-18 have contrasting roles in the tumor milieu. IL-1β induces fever, promotes T cell survival and polarization of Th1, Th2 and Th17

cells, and mediates leukocyte migration[36]. IL-18 can cooperate with IL-12 toward Th1 polarization and activation of NK cells, and can also promote Th17 responses in the presence of IL-23[37]. In the absence of IL-12 and IL-23, IL-18 can vice versa lead to a Th2-bias[37]. Therefore, depending on the microenvironment, IL-1β and IL-18 can facilitate tumor-associated inflammation, rendering the tumor stroma carcinogenic via the release of trophic factors such as fibroblast growth factor 2 (FGF2) and VEGF which allow malignant cells, cancer-associated fibroblasts (CAFs) and endothelial cells to fuel and foster tumor cell survival and invasiveness[38]. In addition, IL-1β induces IL-6, whose pro-tumorigenic activity is mediated through the activation of STAT-3 in an IL-6 receptor (IL-6R)-dependent manner[39]. Similarly, IL-1β can induce TNF-α synthesis/release which in some circumstances can act as an anti-tumor factor[40], but in others can participate to the recruitment of suppressive immune cells, such as myeloid-derived suppressor cells (MDSCs), which favor the neoplastic growth and progression[41]. In support of the pro-tumorigenic potential of IL-1β, the inoculation of lung cancer cells engineered to express higher levels of this cytokine results in higher aggressiveness and dissemination[42]. Moreover, in transgenic mice parietal cells of the stomach able to constitutively secret high level of IL-1β, spontaneously developed gastric inflammation and tumorigenesis correlated to the recruitment of MDSCs, through an IL-1R/NF-κB-dependent pathway[43]. IL-1β secreted by ovarian cancer cells suppresses p53 protein expression in CAF strengthening the pro-tumorigenic inflammatory microenvironment[44].

Another mechanism by which the inflammasome may contribute to immune responses is the secretion of other pro-inflammatory cytokines, such as HMGB1, considered as an endogenous DAMP, and to the induction of growth factors such as FGF2[45], via as yet unclarified biochemical mechanisms. Caspase-1 is essential for FGF2 secretion by macrophages[46]. Unlike IL-1β and

IL-18, IL-1α, HMGB1 and FGF2 are not processed by caspase-1[46–48], suggesting an indirect activity of the inflammasome in the regulation of these unconventional proteins. The activation of fibroblast growth factor receptor 1 (FGFR1) in mammary epithelial cells resulted in increased expression of inflammatory mediators, particularly IL-1β, which cooperate to promote hyperplastic lesions in the mammary gland[49]. In addition, inflammasome-dependent IL-1β exerted pro-carcinogenic activity by suppressing NK and T cell-mediated antitumor actions and immune-editing in a mouse model of carcinogen-induced sarcoma and metastatic melanoma[50]. It is important to note, though, that these proteins might directly depend on caspase-1-mediated pyroptosis. In this context, an intriguing role was identified for caspase-11, which is engaged in the non-canonical inflammasome molecular pathways[51]. While caspase-11 is not critical for caspase-1 activation upon LPS stimulation, it can mediate the activation of the NLRP3 inflammasome during endotoxemia[17,52] and lead to caspase-1 maturation and IL-1β release from infected macrophages. In support of this notion, caspase-11-deficient mice produce lower levels of IL-1β and IL-18[52]. In addition, LPS stimulation fails to secrete normal levels of IL-1β in NLRP3, ASC and caspase-1 deficient macrophages, implying a cross-talk between caspase-1 and caspase-11 activity[53]. Therefore, the engagement of caspase-11 can induce inflammasome-dependent caspase-1 activation and inflammasome-independent pyroptosis-like cell death, via the release of such 'alarmins' as IL-1α and HMGB1[17]. However, little is known about caspase-11 inducer/s. TLR4/TRIF-dependent type I IFN production has been reported to promote caspase-11 activation downstream of NLRP3 activation[54,55]. On the other hand, though, type I IFNs can induce iNOS expression, leading in turn to nytrosilation and thus inactivation of NLRP3[56,57].

Anti-tumor activity of the inflammasome. In sharp contrast, several studies have highlighted an anti-carcinogenic role for inflammasome; particularly, it had a protective role in preventing tumor formation in a Dextran Sodium Sulphate-Azoxymethane (DSS-AOM) mouse model of colon carcinoma[58,59], and its absence increased pro-inflammatory cytokines levels, ERK, NF-κB and STAT3 activation. Similarly, IL-18 was shown to mediate anti-cancer effects in a mouse model of sarcoma and melanoma, involving NK cell-dependent anticancer response[60].

Despite conflicting opinions, there is growing evidence that inflammasome and its products have an essential role in stimulating adaptive immune responses, such as those involved in anticancer immunosurveillance[13]. Indeed, it was also demonstrated that the inflammasome and subsequent IL-1β priming of T cells is critical for immune-mediated eradication of tumors following chemotherapy[61]. Nevertheless, IL-1β was demonstrated to facilitate an immunesuppressive/tolerant response that in the tumor microenvironment reflects into cancer progression.

Therefore, it is obvious to speculate that the inflammasomes have pleiotropic and sometimes contrasting roles in multiple facets of oncogenesis: they positively affect cell-autonomous death pathways and anticancer immunosurveillance, but they also stimulate autocrine or paracrine processes that favor carcinogenic inflammation, tumor growth, metastasis and angiogenesis. To date, the inflammasome involvement represents a double edged sword in that it still remains to be understood if its activation during the neoplastic transformation/cells proliferation or pharmacological treatment can be a benefit or not for the patient.

Aim of the research project. Based on these notions, according to which the inflammasome may influence the formation, progression and therapeutic response of cancer through its contribution to tissue homeostasis, inflammation and immune responses[13], the aim of my PhD project was to investigate inflammasome-dependent pathway/s involved in lung cancer establishment and progression. In particular we tried to understand the involvement of the canonical and non-canonical inflammasome-dependent pathways in lung cancer establishment and progression. We pursued our goal by following specific aims:

1st year: investigate the role of canonical inflammasome in the malignant transformation/progression of lung cancer focusing on immunosuppressive response/s, responsible of oncogenesis;

2nd year: evaluate the role of non-canonical, caspase-8-dependent inflammasome in lung carcinogenesis;

3rd year: evaluate the role of non-canonical, caspase-11-dependent inflammasome in lung carcinogenesis.

CHAPTER 1

Caspase-1-dependent canonical inflammasome was involved in lung cancer establishment and progression.

1.1 Introduction

Caspase-1, formerly called as IL-1β converting enzyme (ICE), is a member of a family of nine cysteine proteases that seem to be uniquely involved in the inflammatory response by cleaving the precursors of IL-1β, IL-18, and IL-33. Indeed, the rate-limiting step in IL-1β or IL-18-dependent inflammation is the activation of caspase-1[14]. Inactive pro-caspase-1 is converted to an active enzyme following homodimerization that allows an autocatalytic cleavage that releases the active enzyme composed of two large and two small subunits. The autocatalysis of pro-caspase-1 to active caspase-1 is tightly controlled by the inflammasome assembly[62]. Caspase-1 activation can also induce pyroptosis, an inflammatory cell death that is accompanied by the release of IL-1β and IL-18 which elicits local inflammation[63]. Although various studies demonstrated that an increased concentration of IL-1β protein in tumor tissues was associated to poor prognosis for cancer patients[64], the function of inflammasomes in tumor growth and metastasis remains controversial. Published studies mainly use AOM/DSS-induced colon cancer as an animal model to evaluate the involvement of inflammasomes in cancer[59,60]. Results from those studies indicate that inflammasome components (i.e. NLRs) provide protections against tumorigenesis in colitis-associated colon cancer, as mice deficient for inflammasomes, including NLRP3, NLRP12, NLRC4 and caspase-1, have increased tumorigenesis in the AOM/DSS-induced colon cancer animal model due to lower cell death rate[58]. However, in other types of cancer, such as melanoma and mesothelioma, caspase-1-dependent canonical inflammasomes and IL-1β have been shown to enhance tumor growth[64]. Furthermore, blocking IL-1R with IL-1R antagonist (IL-1Ra) inhibits tumor

progression accompanied by decreased myeloid cells recruitment in preclinical breast cancer models[64]. According to the lack of data in lung cancer and to the conflicting results reported in literature, we focused on the role of caspase-1-dependent canonical inflammasomes during lung cancer establishment and progression using human non-small cell lung cancer (NSCLC) tissues and a mouse model of carcinogen-induced lung cancer.

We find that caspase-1 is in its active form in both human and mouse lung cancer tissues compared to non cancerous tissues. In support, the pharmacological inhibition of caspase-1 by means of a specific inhibitor (Ac-Y-Vad) significantly decreases lung tumor development in mice. This effect is associated to a reduced release of pro-inflammatory cytokines and recruitment of immunosuppressive cells to the lung of tumor-bearing mice. These data demonstrate that caspase-1 activation occurs during lung carcinogenesis in both humans and in an experimental mouse model.

1.2 Materials and Methods

Human Samples. Human tissue of NSCLC and non-cancerous lung tissues were obtained from patients undergoing thoracic surgery at the Hospital of Salerno "San Giovanni di Dio e Ruggi D'Aragona". The protocol was approved by Ethics Committee and patients signed the informed consent. The mean age of patients was 60±10 years and equally distributed for gender. We excluded subjects with metastatic lung carcinoma.

Cancerous tissues (LC) were obtained from the tumor mass whereas 'non–lung cancerous tissues' (NLC), derived from a distant portion of the tumor mass. Human samples were collected within 12 hours from the surgery and processed within the following 3 hours.

Mice. Female C57Bl/6 mice (6-8 weeks; Charles River, Lecco, Italy) were maintained in specific pathogen-free conditions at the University of Salerno, Department of Pharmacy. All animal experiments were performed under protocols that followed the Italian (DL No. 26/2014) and European Community Council for Animal Care (Directive 2010/63/EU) on the protection of animals used for scientific purposes.

Experimental Protocol. To investigate the role of caspase-1-dependent canonical inflammasome in the establishment/progression of lung carcinoma, a carcinogen-induced mouse model of lung cancer was used. Under isoflurane anaesthesia, *N*-methyl-*N*-nitrosourea (NMU), an alkylating and mutagen agent[65], was intratracheally (i.t.) instilled into C57Bl/6 mice (female; 6-8 weeks of age) for three consecutive weeks, according to the following protocol: 50μg/mouse (in 10 μl of saline) at t=0 followed by other two administrations of 10μg/mouse (in 10 μl of saline) at t=7 days and t=14 days (Figure 4).

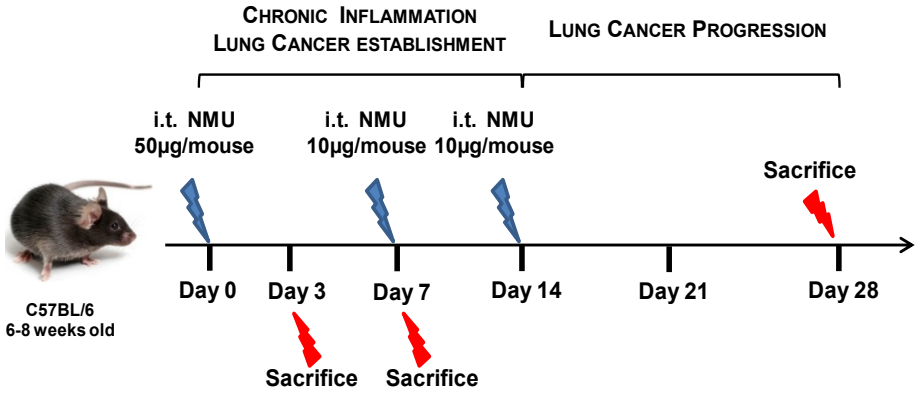

Figure 4. Experimental protocol: C57Bl/6 mice (female, 6-8 weeks of age) were intratracheally (i.t.) exposed to *N*-methyl-*N*-nitrosourea (NMU) for three consecutive weeks at the dose of 50µg/mouse at week 1 (t=0), and at the dose of 10µg/mouse at week 2 (t=7) and 3 (t=14). Mice were sacrificed at different time points: 3, 7 and 28 days post the first NMU exposure.

In an initial set of experiments, immunofluorescence analyses were performed on left lung criosections obtained from NMU-exposed mice in order to define first the establishment of the lung tumor mass and then to indentify tumor hystotype generated. Left lung criosections were analysed for the presence of Ki-67 (Dako, Cambridge, UK), K-Ras, p63 and cytokeratin 5 (K5) (Santa Cruz technologies, CA, USA). Alexa-Fluor-555 and FITC staining were used to identify the above biological targets. Images were observed by means of Carl Zeiss confocal microscopy (magnification: 40X) (Figure 5). According to a blind pathologist, the lungs of NMU-exposed mice sacrificed at 7 days showed inflamed lesions, whereas hyperplastic/tumor lesions were visible starting at 4 weeks in the lung of NMU-treated mice[66]. In our experimental conditions, the incidence of lung tumor in mice was around 80% at 4 weeks

post the first NMU exposure. Tumor incidence was analysed as the percentage of tumor formation of each lung compared to the average of all tumor area/total lung area of n=39 mice exposed to NMU. Moreover, NMU-exposed mice showed that the tumor was adenocarcinoma-like according to immunofluorescence analyses (Figure 5) which showed that lung tumor cells were Ki-67^{+}K5^{+}p63^{-}, similarly to the human lung adenocarcinoma[67].

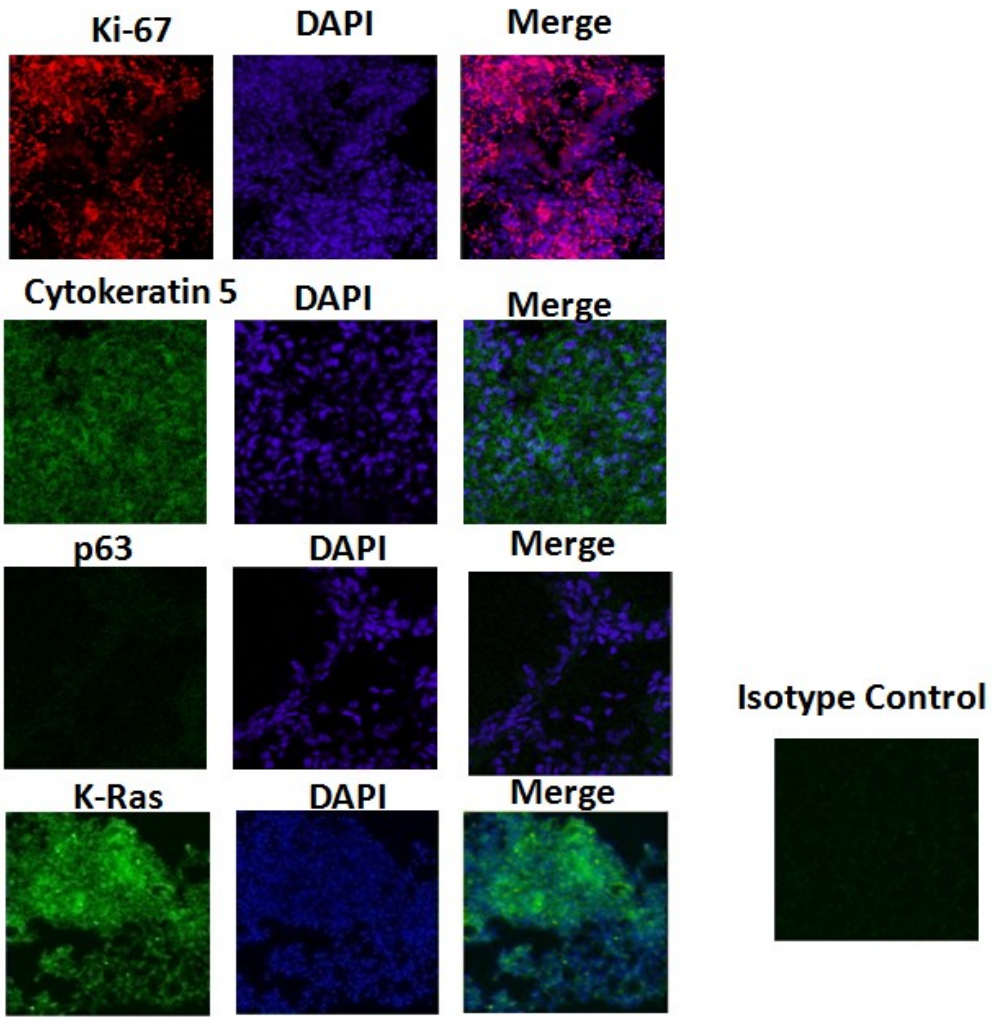

Figure 5. Representative pictures of Ki-67^{+}K5^{+}p63^{-}K-Ras^{+} tumor cells in the lung of NMU-exposed mice.

Mice were divided into the following groups: 1. Naïve, non-treated, mice; 2. Control (CTR), i.t. instilled with NMU; 3. NMU-instilled mice treated with Ac-Y-Vad (y-VAD; 1-10μg/mouse), a specific caspase-1 inhibitor.

Animals were sacrificed at different time points (3, 7 and 28 days) to study the involvement of caspase-1 during lung cancer development and progression. Bronchoalveolar lavage (BAL) fluid was obtained by using a solution of PBS (0.5 mL) and EDTA (0.5 mM) to measure cytokine levels. Lungs were excised for staining and biochemical tests. Left lung lobes were embedded into OCT medium to perform Haematoxylin & Eosin (H&E) staining to evaluate the tumor lesion, expressed as ratio of Tumor Lesion Area/Total Lung Area, calculated with ImageJ software (NIH, USA); whereas, right lung lobes were divided into two parts: one part underwent digestion with 1U/mL of collagenase to perform flow cytometry analyses, and the other underwent homogenization to perform ELISAs and western blotting analyses.

Flow Cytometry Analysis. Cell suspensions, from digested lungs, were passed through 70 μm cell strainers and red blood cells were lysed. To investigate the immune infiltrates into the lung of tumor-bearing mice, lung cell suspensions were labelled with specific antibodies: CD11b, Gr-1, CD4, CD25, FoxP3.

Cytokine measurements. IL-6, IL-1α, IL-1β, IL-18, IL-33, TNF-α and IL-10 were measured in lung homogenates (expressed as pg/mg of proteins) and in BAL (expressed as pg/mL) by using commercially available ELISAs (eBioscience, CA, USA; R&D Systems, London, UK).

Western blot analysis. Lung homogenates were used to examine the expression of caspase-1 active form (20-10 kDa) vs the precursor form (46 kDa) (Santa Cruz Technologies, CA, USA).

Statistical analysis. Results are expressed as median±interquartile range and analysed with Mann Whitney test for non parametric data. P values less than 0.05 were considered as significant.

1.3 Results

1.3.1 Caspase-1 was active in both humans and mice during lung carcinogenesis.

The active form of caspase-1 is involved in the release of the mature IL-1β and IL-18, highly present in the serum and lung of cancer patients[18]. Because of the conflicting data in literature about the pro- or anti-tumorigenic role of caspase-1, and thus of the inflammasome, in carcinogenesis and due to the lack of an experimental carcinogen-induced mouse model for lung cancer, our main goal was to first establish a mouse model of carcinogen-induced lung cancer and then understand inflammasome involvement in lung carcinogenesis, by comparing the experimental model to human samples of NSCLC. As already described in materials and methods section of this Chapter (paragraph 1.2), NMU-exposed mice showed an adenocarcinoma-like lung lesion, which established at 28 days (4 weeks) after the first NMU exposure (Figure 5), preceded by lung inflammation due to the exposure of the carcinogen, as also demonstrated by the release of pro-inflammatory cytokines at 7 days (i.e. IL-6 and TNF-a, Figure 10A and 10B below). In order to understand the involvement of caspase-1 in a time-dependent manner in lung carcinogenesis, western blotting analyses were performed on lung homogenates obtained from NMU-exposed mice sacrificed at 3-7-28 days (Figure 6). Caspase-1 was present in the lung of NMU-exposed at 3, 7 and 28 days compared to naïve mice (Figure 6A and 6B). To note, the expression of the active form was significantly higher at 7 days compared to 3 and 28 days post NMU exposure (Figure 6B), implying the activation of the enzyme post the genotoxic injury, which led to an inflammatory response that to the establishment of lung tumor mass starting at 28 days. In order to verify whether the activation of caspase-1 and thus of the canonical pathway of the inflammasome occurred in human lung cancer, we used lung tissues of non-cancerous (NLC) and cancerous lesions (LC), obtained from patients

undergoing thoracic surgery. Similarly to mice, caspase-1 was in its active form (20 kDa) in human LC tissues (n=7) but not in NLC tissues (n=7) (Figure 7A and 7B).

Taken together, these results demonstrate that caspase-1 is in its active form in both human lung cancer samples and in a mouse model of lung carcinoma, implying that rather than inducing cell death or defense mechanisms, as described in literature[63], it is involved in tumor proliferation.

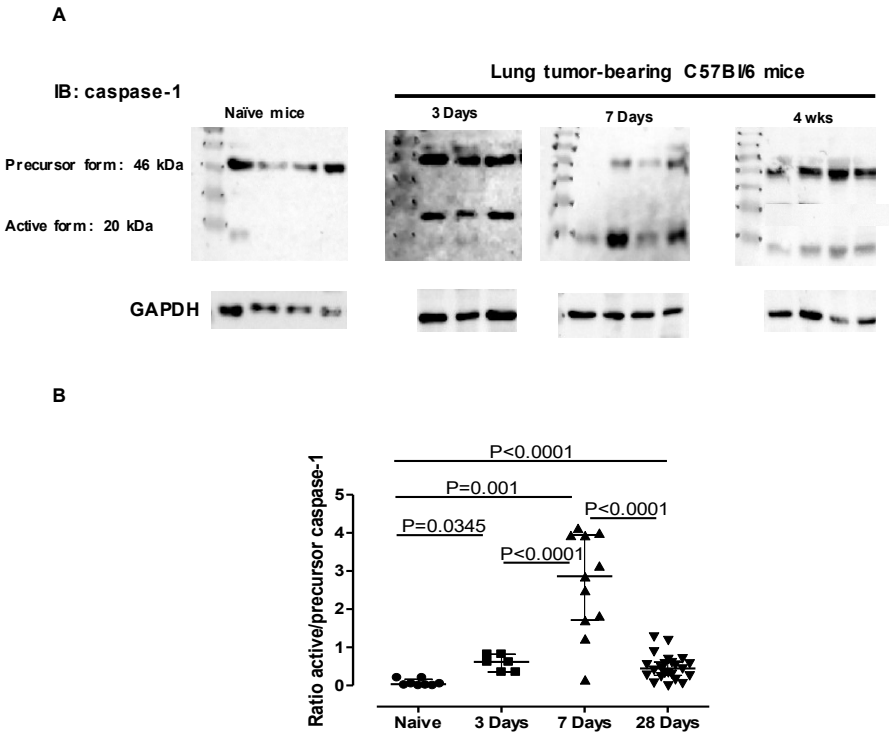

Figure 6. Caspase-1 was active in the lung of tumor-bearing mice exposed to NMU. A) Western blot analyses, performed on lung homogenates obtained from NMU-exposed mice, showed higher levels of the active form of caspase-1 (20 kDa) at 3, 7 and 28 days compared to naïve mice. **B)** Quantitative analysis (ratio active vs precursor form of caspase-1) showed higher levels of the active form of caspase-1 in lung tumor-bearing mice.
Data are expressed as median±interquartile range and analysed with Mann Whitney test for non-parametric data.

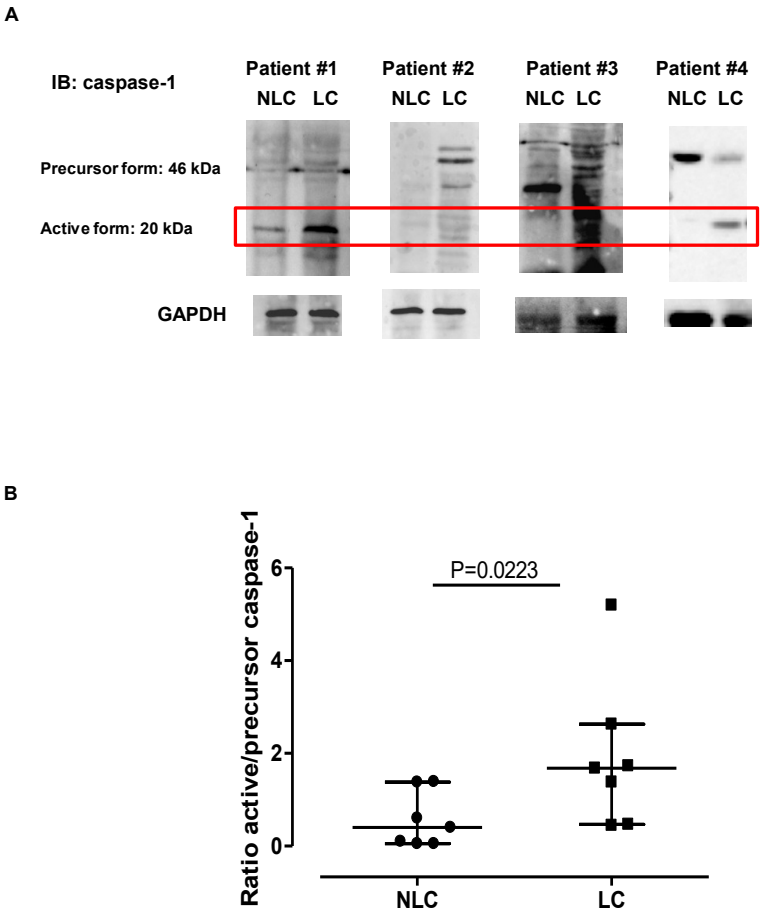

Figure 7. Caspase-1 was active in human samples of non-small cell lung cancer (NSCLC) patients. A) Representative western blotting analyses performed on human samples of NSCLC showed caspase-1 in its active form in LC but not in NLC tissues. **B)** Data were stated as ratio of the active (20 kDa) vs precursor (46 kDa) form of caspase-1, expressed as median±interquartile range and analysed with Mann Whitney test for non-parametric data.

1.3.2 Pharmacological inhibition of caspase-1 reduced lung cancer progression in mice modulating the pro-inflammatory pathways.

In order to evaluate whether the activation of caspase-1 was pivotal for lung cancer establishment/progression, we used a specific pharmacological inhibitor, Ac-Y-Vad (y-VAD). y-VAD was administrated, intraperitoneally (i.p.), at 1 or 10μg/mouse/twice/week from time 0 after NMU instillation up to the time of sacrifice of mice (Figure 8A). We observed that the administration of the low dose of y-VAD (1μg/mouse) did not alter tumor lesion at 28 days (4 weeks) (Figure 8B). In sharp contrast, the higher dose of y-VAD (10μg/mouse) significantly reduced lung tumor lesion compared to control mice (Figure 8B), suggesting an important role of caspase-1 during lung tumor establishment/progression. With these results, a question was obvious: what happens if caspase-1 is inhibited when the tumor is already formed? Therefore, another set of experiment was set up: mice were treated with y-VAD (1-10μg/mouse) twice a week starting from day 28 after the first NMU instillation, when the tumor lesion was already formed. Mice were sacrificed 7 days after y-VAD administration (35 days). In this type of experiments, the sole administration of y-VAD 10μg/mouse, but not of y-VAD 1μg/mouse, significantly reduced lung tumor lesions compared to control mice (Figure 8C). To further prove, the relevance of caspase-1, we used caspase-1 ko mice, which were later identified as caspase-1/11 double knock-out (DKO) mice. The genetic absence of caspase-1 significantly reduced tumor lesions in the lung of NMU-exposed mice at 28 days post the first NMU exposure (Figure 9). However, it is to note that the tumor lesion in DKO was 0.048±0.0074 (Figure 9) versus 0.077±0.076 (Figure 8B) in mice treated with y-VAD (10μg/mouse), implying the involvement of another orchestrator in lung tumor environment, as described in Chapter 3.

In our preliminary data we observed that caspase-1 was in its active form at earlier time points as 3 and 7 days, experimental time points during which

lung was characterized by an inflamed status (materials and methods section, paragraph 1.2) after the exposure to the alkylating/genotoxic agent NMU. Therefore, to better understand the role of caspase-1 in tumor-related inflammation, we went on by analyzing the release of pro-inflammatory cytokines in the tumor microenvironment by using homogenized lung tissues and BAL from NMU-exposed mice. The administration of y-VAD 10μg/mouse significantly reduced the release of the pro-inflammatory IL-6 (Figure 10A) and TNF-α (Figure 10B). In sharp contrast, the direct inflammasome-dependent cytokines IL-1β and IL-18 did not show any reduction after y-VAD treatment. In particular, IL-1β levels were significantly increased at 28 days (Figure 10C), and IL-18 levels (Figure 10D) were unchanged compared to control as revealed in the BAL obtained from tumor-bearing mice compared to the control group. In addition, the levels of IL-1α (Figure 10E) and IL-33 (Figure 10F) were significantly reduced in y-VAD-treated than in control lung homogenates obtained from tumor-bearing mice. To note, IL-1α and IL-33 are not strictly dependent on caspase-1 activation[54].
Taken together, our data show that caspase-1 is involved in the amplification of the inflammatory process associated to lung cancer establishment and progression in mice. However, the canonical inflammasome-dependent pathway is not altered in terms of IL-1β and IL-18 release, after the inhibition of caspase-1 in mice, instead associated to lower lung tumor burden (Figure 8B and 8C).

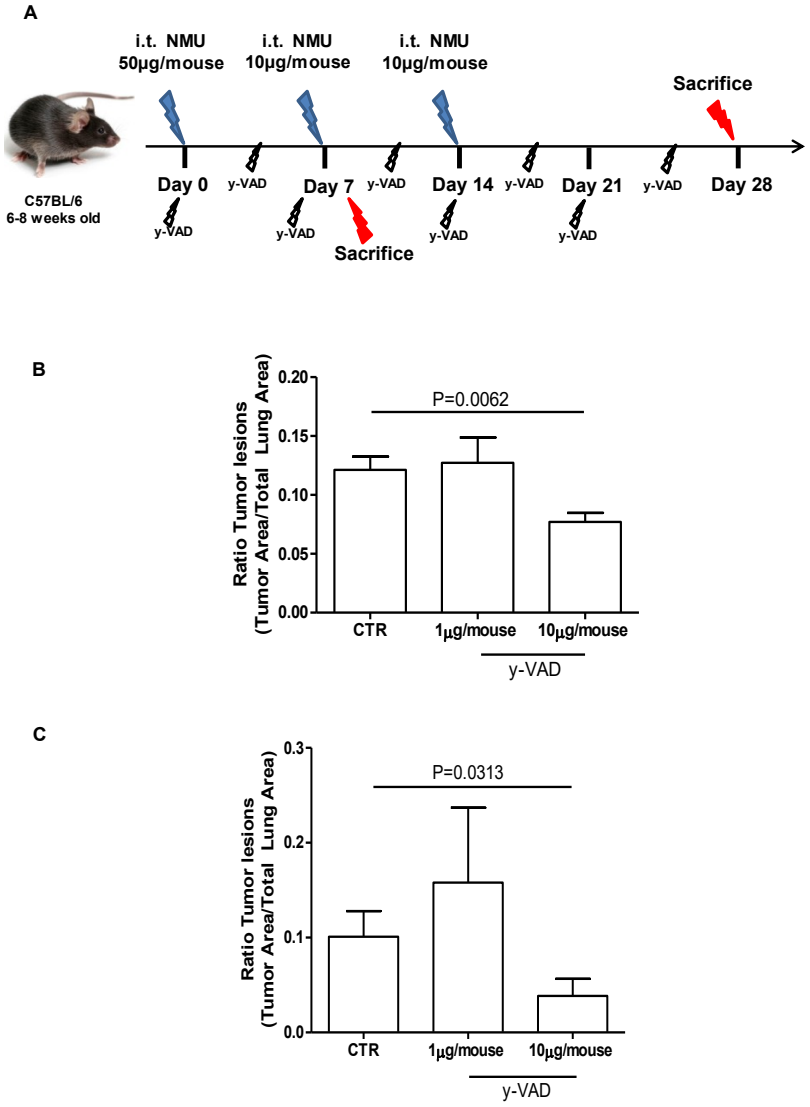

Figure 8. Pharmacological inhibition of caspase-1 reduced lung cancer progression in mice. A) Experimental protocol. **B)** The administration of y-VAD 10µg/mouse/twice/week from time 0 after NMU instillation, significantly reduced lung tumor formation at 28 days compared to control mice. **C)** In another set of experiments, mice were treated with PBS (CTR) or y-VAD 1-10µg/mouse at 28 days after the first exposure to NMU. y-VAD 10µg/mouse, but not y-VAD 1µg/mouse, significantly reduced lung tumor lesion compared to control mice. Tumor lesion was expressed as ratio between tumor area and total lung area. Data are expressed as median±interquartile range and analysed with Mann Whitney test for non-parametric data.

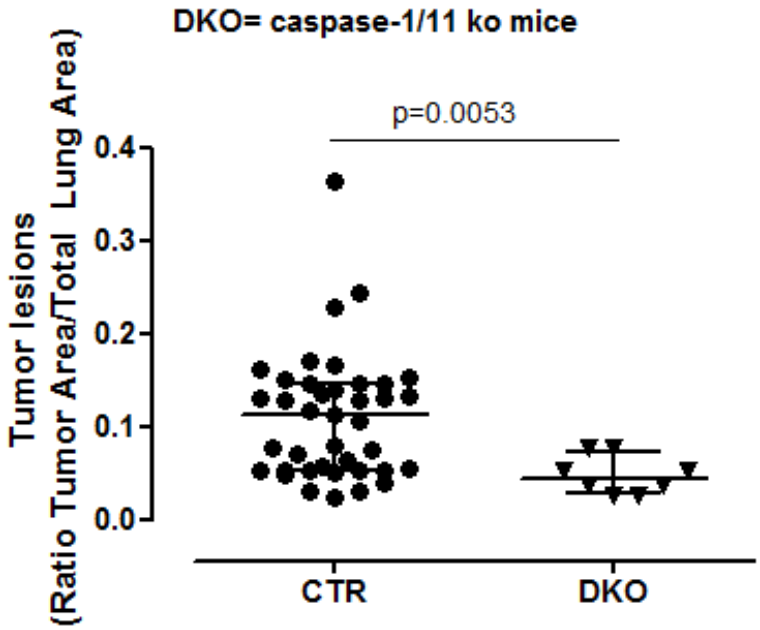

Figure 9. The genetic absence of caspase-1 reduced lung cancer establishment/progression in mice. Caspase-1/11 knock-out (ko), identified as double ko (DKO), were exposed to NMU as for CTR mice. DKO mice showed lower tumor lesion than CTR mice. Data are expressed as median±interquartile range and analyzed by Mann Whitney test as for non-parametric data.

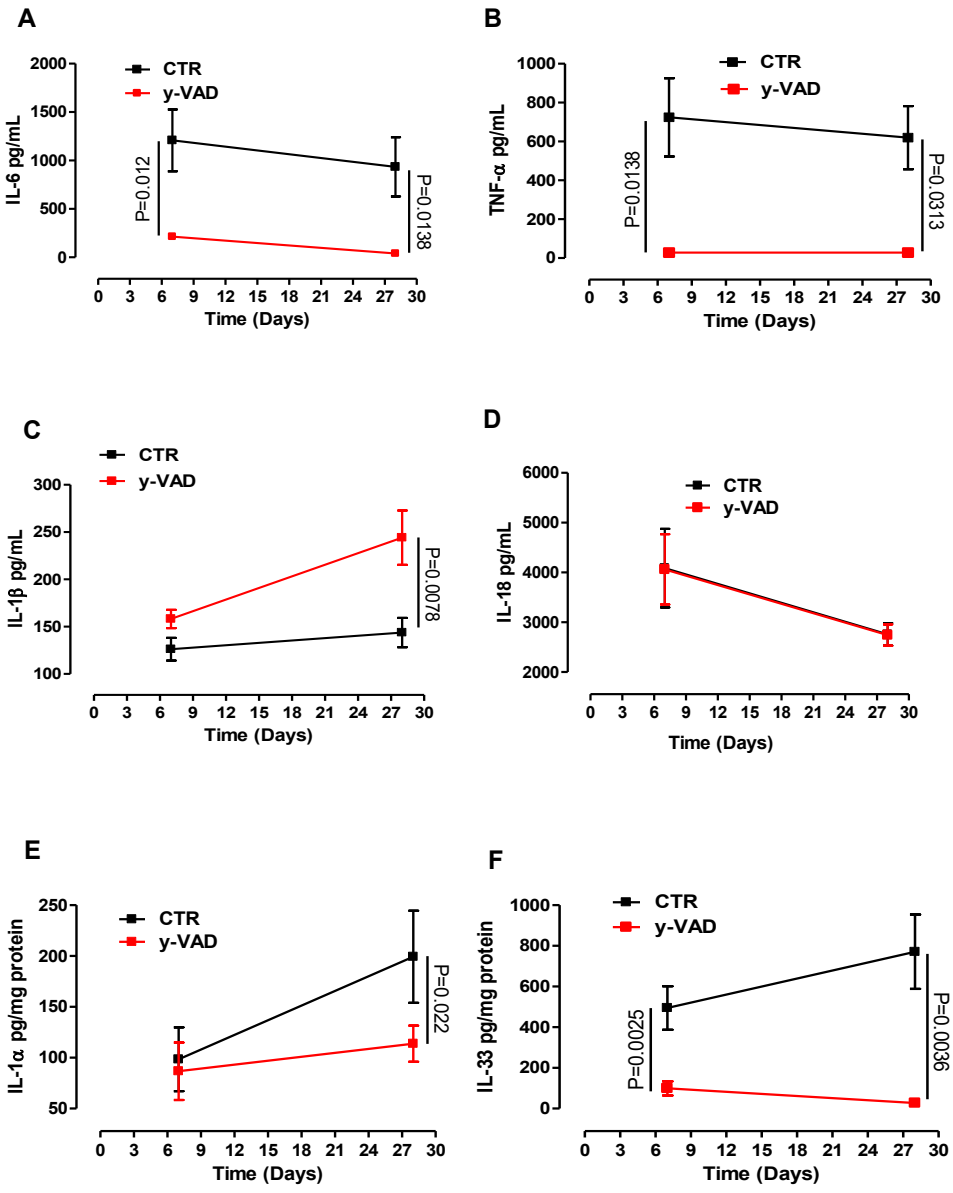

Figure 10. The pharmacological inhibition of caspase-1 reduced pro-inflammatory cytokine release, but not inflammasome-dependent cytokines. Broncho-aleveolar lavage (BAL) fluid was harvested from NMU-exposed mice at 7 and 28 days. The pharmacological inhibition of capase-1 by means of y-VAD 10µg/mouse significantly reduced the levels of IL-6 (**A**) and TNF-α (**B**), but not of IL-1β (**C**) and IL-18 (**D**) in the BAL of lung tumor-bearing mice. Similarly, the levels of IL-1α (**E**) and IL-33 (**F**) were significantly reduced in lung homogenates obtained from control and y-VAD-treated NMU-exposed mice. Data are expressed as median±interquartile range and analyzed by Mann Whitney test for non-parametric data.

1.3.3 Inhibition of caspase-1 modulated the immune-suppressive microenvironment in the lung of tumor-bearing mice.

Growing evidence indicates that the immune system is pivotal for malignant disease control[68]. The suppressive immunity has been acknowledged for its contribution to cancer development and progression[68,69], due to the immune system failure to attack malignant cells as 'non-self'. Based on this concept, we moved on by analyzing the immune lung tumor microenvironment after caspase-1 inhibition. The inhibition of caspase-1 by means of y-VAD (10μg/mouse) reduced the recruitment to the lung of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs: identified as $CD11b^{high}Gr\text{-}1^{high}$ cells) and T regulatory cells (Treg: identified as $CD4^{+}CD25^{+}FoxP3^{+}$ cells) compared to the control group (Figure 11A and 11B respectively). These results were consistent with the decrease of tumor lesion observed in y-VAD-treated mice. However, the levels of IL-10, immunosuppressive cytokine, in the BAL of both control and y-VAD-treated mice did not show any statistically significant difference, though higher in CTR mice than y-VAD-treated mice (Figure 11C).

Taken altogether, these data suggest that the pharmacological inhibition of caspase-1 interferes with the recruitment of immune-suppressive cells to the lung in tumor-bearing mice, implying a critical role for caspase-1 during lung tumor immunoediting.

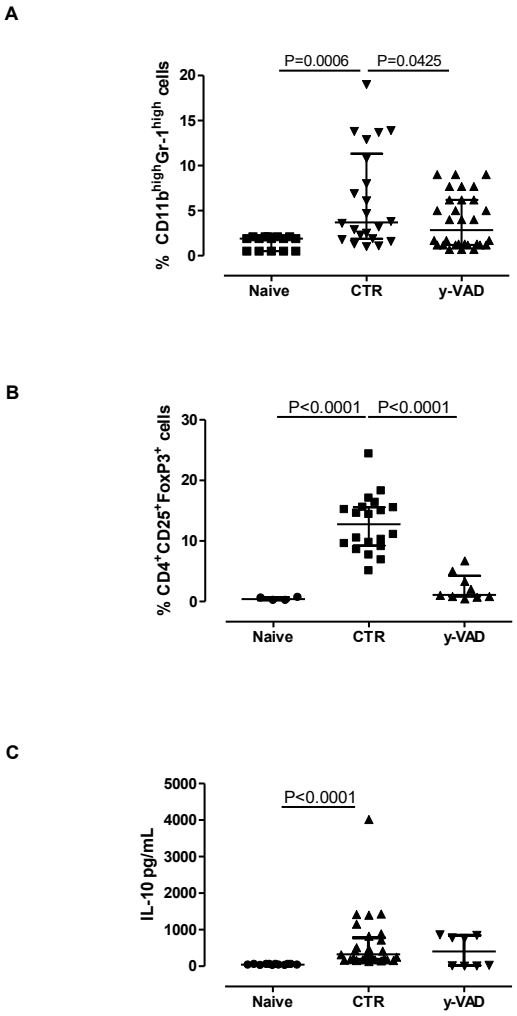

Figure 11. The pharmacological inhibition of caspase-1 modulated the immunesuppressive microenvironment in the lung of tumor-bearing mice. The pharmacological inhibition of caspase-1 significantly reduced the percentage of myeloid-derived suppressor cells (MDSC: identified as $CD11b^{high}Gr\text{-}1^{high}$ cells) (**A**), and T regulatory cells (Treg: identified as $CD4^{+}CD25^{+}FoxP3^{+}$ cells) (**B**). IL-10 levels in the BAL of control (CTR) and y-VAD-treated lung tumor-bearing mice did not show statistically significant differences, but they were higher than naïve mice (**C**). Data are expressed as median±interquartile range, analysed with Mann Whitney test for non-parametric data.

1.4 Conclusions

The activation of the inflammasome in the context of tumor microenvironment has yielded to conflicting results, and details of its involvement in lung cancer are very ill-defined. Here we show that caspase-1 is involved in lung inflammation and then lung tumor formation/progression in mice. Similarly, human samples of lung cancer tissues show that caspase-1 is in its active form, implying the activation of the inflammasome. In support, the pharmacological inhibition of caspase-1 significantly reduces both the establishment and the progression of the lung tumor area in mice.

The incidence of lung tumor is associated to chronic infections, inhaled pollutants, cigarette smoking[21,70,71]. According to the sterile inflammation theory, sterile, noninfectious insults, such as reactive oxygen species (ROS), oxidized and/or methylated DNA, HMGB1, heat shock proteins, ATP, generally identified as DAMPs, can induce chronic inflammation[26,72]. All these stimuli can behave as tumor promoters, which underlying activity is the induction of chronic inflammation that, rather than providing a protective response to loss of tissue homeostasis, can aberrantly facilitate tumor development. These insults, both endogenous and exogenous, are sensed by the multimeric complex called inflammasome[14]. In our human samples, that derived majorly from smokers, we observed that caspase-1 was in its active form[73]. To date, these patients showed higher content of mitochondrial ROS in peripheral blood-derived mononuclear cells (PBMCs) (data not shown), following particulate matter exposure[73]. In our published data[73], we showed that the ultrafine particles (UFP) induced the activation of the inflammasome in a NLRP3-caspase-1-dependent manner. These latter data, correlated to the data in this thesis about the activation of caspase-1 in the lung tumor lesion of lung cancer patients, suggest that the activation of the inflammasome plays a role in lung cancer, despite what reported in literature about its anti-tumorigenic activity. Dr. Allen and colleagues showed that caspase-1 ko mice

had increased the susceptibility and number of colon polyps in a DSS-AOM mouse model of colon carcinoma, showing that the inflammasome has a protective role in preventing tumor formation in the colon[74]. This effect was ascribed to the induction of cell death after caspase-1 activation. In sharp contrast, in our experimental and human lung cancer samples we did not observe the induction of cell death via apoptosis. We analysed both the levels of the active form of caspase-3 and caspase-9 that are involved in cell death. We did not observe any activation of these two caspases and even more, we observed no reduction of the anti-apoptotic protein Bcl-2 (data not shown), implying that the activation of caspase-1 is not involved in cell death in lung cancer establishment and progression in both mice and humans.

To prove caspase-1 as of potential therapeutic target, we performed experiments during which caspase-1 was pharmacologically inhibited by a commercially available specific inhibitor, y-VAD. We show that the pharmacological inhibition of caspase-1 by means of y-VAD significantly reduces lung cancer outgrowth associated to lower pro-inflammatory response and to a reduced lung recruitment of immunosuppressive cells. However two main issues raised from these experiments: 1. the genetic absence of caspase-1 further reduced the tumor area in NMU-exposed mice which was 0.048±0.0074 versus the tumor area observed in y-VAD treated mice, which was 0.077±0.076; 2. the pro-inflammatory pattern (i.e. TNF-α and IL-6, IL-1α and IL-33), non strictly dependent on inflammasome activation was reduced in y-VAD-treated NMU-exposed mice, but instead IL-1β and IL-18, strictly dependent from the inflammasome activation were not altered.

These two issues have a common explanation. Caspase-1 ko mice were discovered as double ko mice in that they lack both caspase-1 and caspase-11[17], implying that another orchestrator upstream caspase-1 was involved. In the meanwhile, a paper from Dr Kannenganti's group was published, showing that the caspase-8-dependent non-canonical inflammasome activation was

responsible of the activation of IL-1β and IL-18 in bone marrow-derived macrophages[75]. Therefore, two questions were opened: is caspase-8 active in our samples and is it upstream responsible for caspase-1 activation? Or is caspase-11 upstream caspase-1 activation? These two questions were approached and tried to be answered in Chapter 2 and 3.

Moreover, the immunesuppressive environment is altered in y-VAD-treated vs CTR mice in that MDSCs and Treg are lower recruited to the lung of NMU-exposed mice, although we do not detect differences in IL-10 levels, most probably due to partial reduction of inflammasome pathway involved in lung carcinogenesis. In support, we already proved that human lung tumor lesions are populated by immunesuppressive cells[76,77] in which the activation of the inflammasome plays a pivotal role for the immunesuppression/tumor progression axis. Of note, oxidized DNA, highly relevant during tumor initiation/progression, potentiates stimulator of IFN I genes (STING)-dependent signaling pathways[78], which is responsible for immune-regulatory/suppressive responses via the activity of indoleamine-2,3-dioxygenase (IDO) in DC[78] and for the recruitment and differentiation of Treg in tumor masses[79]. Similarly, we proved that plasmacytoid DCs (pDCs) released IL-1α and IL-1β in a caspase-1-dependent manner in human lung tumor lesions, favoring cancer progression[76].

In conclusion, these first data demonstrate that the activation of the inflammasome via caspase-1 is involved in lung tumor establishment and progression, favoring the immunesuppressive environment that allows tumor cells to escape the immune system activity. To date, though, we believe that another orchestrator, upstream caspase-1, governs the inflammasome activation in favor of lung carcinogenesis. This hypothesis has been approached in Chapter 2 and 3.

CHAPTER 2

Caspase-8-dependent non-canonical pathway of inflammasome was involved during lung cancer establishment and progression.

2.1 Introduction

Caspase-8 is an apical caspase which initiates programmed cell death following death receptor ligation[80]. This central role in apoptosis has prompted significant clinical interest in promoting caspase-8 expression and activity as therapeutic opportunity in cancer. Nonetheless, caspase-8 expression is heterogeneous and sometimes even elevated in tumors; therefore, some doubt still remain as to whether its expression correlates with clinical benefits. To note, one of the main mechanism for chemotherapy resistance is associated to the evasion of tumor cells to the apoptotic, non-inflammatory, cell death[81]. Caspase-3 and -9 represent the main caspase effectors that lead to the apoptotic cell death[82]. However, other caspases underlying apoptosis-mediated cell death occur. In this scenario, caspase-8 is one of the upstream mediator of cell death which activation is related to TNF-like cytokines, such as TNFα, Fas ligand (FasL; CD95)[82]. Caspase-8 is a cysteinyl protease which is activated through the formation of a death-inducing signaling complex when death receptors are complexed to their specific ligands. Engagement of death receptors such as CD95, TNF-related apoptosis-inducing ligand (TRAIL) and TNF receptor 1 (TNFR1) results in recruitment of caspase-8 and its adaptor Fas-Associated protein with Death Domain (FADD) to initiate an apoptosis cascade that leads to caspase-3 (extrinsic) dependent and caspase-9 (intrinsic) dependent pathways[82]. However, although the fine relation between apoptosis and inflammation has been separated for years, caspase-8 has been recently demonstrated to mediate the canonical and noncanonical NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome priming and activation[75]. The activation of caspase-8 induced inflammasome-dependent

IL-1β release from macrophages in a canonical, but also in a non canonical, manner by promoting the activation of caspase-1. Indeed, caspase-1/11 knockout macrophages were still able to release IL-1β under infection conditions[83]. In this context, though, caspase-8 inhibits the kinase RIP3, which is involved in necrosis and predominates on caspase-8-induced apoptosis[84,85]. Notably, mice deficient of caspase-8 were embrionic lethal, explaining the important role of caspase-8 to alter the necrotic cell death, but also to induce proliferation, as demonstrated in T lymphocytes[86]. Activated T lymphocytes were induced to proliferation under caspase-8 control that led to NF-kB and MAPK activation. In this scenario, caspase-8 can therefore be categorized as both apoptotic/non-inflammatory caspase and pro-inflammatory caspase together with caspase-1 and caspase-11.

Caspase-8 was inactivated in a variety of human cancers[87], which may promote tumor progression as well as resistance to current treatment approaches. Shivapurkar and colleagues, described that caspase-8 was silenced in small cell lung cancer (SCLC), suggesting that its absence was responsible for tumor cell chemoresistance under TRAIL stimulation[88]. In contrast, Riley and colleagues[81], demonstrated that pro-caspase-8 was overexpressed in NSCLC and represented a marker for poor patient prognosis. To date, caspase-8 has also been found to play a number of non-apoptotic roles in cells, such as promoting activation NF-κB signaling, regulating autophagy, altering endosomal trafficking and enhancing inflammatory pathways, cellular adhesion and migration. Therefore, based on these concepts, caspase-8, depending upon the specific cellular context, may either potentiate or suppress tumor malignancy.

Therefore, the aim of our study was to understand and clarify the role of caspase-8 in human lung cancer, taking advantage of a mouse model of carcinogen-induced lung cancer.

2.2 Materials and methods

To investigate the role of caspase-8-dependent non-canonical inflammasome in the establishment/progression of lung carcinoma, human samples obtained from lung cancer patients and a mouse model of carcinogen-induced lung cancer were used, as discussed in the materials and methods section in Chapter 1, paragraph 1.2.

Experimental Protocol. Briefly, C57Bl/6 mice (female; 6-8 weeks of age) (Charles River, Lecco, Italy) were exposed to *N*-Methyl *N*-Nitroso Urea (NMU), intratracheally (i.t.), under anaesthesia with isoflurane. Similarly to Chapter 1, we followed the experimental protocol as described in Figure 4 paragraph 1.2. Moreover, in this set of experiments, NMU-exposed mice were intraperitoneally (i.p.) treated with z-IETD-FMK (IE: 0.5µg/mouse), specific inhibitor of caspase-8, twice a week starting from the first exposure to NMU (Figure 14A). In particular, mice were divided into the following groups: 1. Naïve, non-treated, mice; 2. Control (CTR), NMU-instilled; 3. IE, NMU-instilled mice treated with z-IETD-FMK (IE; 0.5µg/mouse) and 4. y-VAD 10+IE, NMU-instilled mice treated with Ac-y-Vad (10µg/mouse)+z-IETD-FMK (0.5µg/mouse).

The animals were sacrificed at different time points (7 and 28 days) to study the involvement of caspase-8 during lung cancer development and progression. Left lung lobes were embedded into OCT medium to perform H&E staining to evaluate the tumor lesion, expressed as Tumor Lesion Area/Total Lung Area. Right lung lobes were homogenized with 1U/mL of collagenase (Sigma Aldrich, Rome, Italy) to evaluate the release of cytokines and immune cells infiltrates to the lung.

Broncho-alveolar fluid (BAL) was collected by means of airway lavage by using a solution of PBS (0.5mL) and EDTA (0.5mM) to measure pro-inflammatory and anti-inflammatory cytokine levels.

Flow Cytometry Analysis. Lungs were isolated and digested as described above. To investigate the immune infiltrates into the lung of tumor-bearing mice, lung cell suspensions were labelled with specific antibodies (CD11c, CD11b, B220, F4/80, Gr-1, PDCA-1, MHC I, MHC II, CD3, CD4, CD25, FoxP3).

Cytokine measurements. IL-6, IL-1α, IL-1β, IL-18, IL-33, TNF-α and IL-10 were measured in lung homogenates (expressed as pg/mg of proteins) and in BAL (expressed as pg/ml of proteins) by using commercially available ELISAs (eBioscience, CA, USA; R&D Systems, London, UK).

Western blot analysis. Lung homogenates were used to examine the expression of caspase-8 active form (22 kDa; Santa Cruz Technologies, CA, USA). Similarly, c-FLIP and Bcl-2 (Santa Cruz Technologies, CA, USA) expression was evaluated.

Statistical analysis. Results are expressed as median±interquartile range and analysed with Mann Whitney test for non-parametric data. P values less than 0.05 were considered significant.

2.3 Results

2.3.1 Caspase-8 was active in both human and mouse samples of lung cancer.

Evasion of apoptosis is considered to be one of the hallmarks of human cancers[8]. Cell death is executed by caspases and several upstream regulatory factors, which direct their proteolytic activity. Often these regulatory factors, in addition to being potent apoptosis inducers, function in cell survival or repair signaling pathways in response to cellular stress. Since caspase-8 presents a key regulator of apoptosis, inactivation of caspase-8 can confer resistance to cell death. Genetic, epigenetic as well as posttranslational changes can contribute to inactivation of caspase-8 in human malignancies[87]. Thus, restoration of caspase-8 function represents a promising strategy to either directly trigger apoptosis in cancer cells or to restore sensitivity to apoptotic stimuli. In order to investigate the role of caspase-8 in lung cancer establishment and progression, human samples of NSCLC were enzymatically digested and tested to evaluate its expression. Western blotting analyses showed that both Non Lung Cancer (NLC) and Lung Cancer (LC) tissues had similar levels of the active form (22 kDa) of caspase-8 compared to the precursor form (55 kDa) (Figure 12A and 12B).

Because of the dichotomy of caspase-8 in human samples, we took advantage of the established carcinogen-induced lung cancer mouse model (Figure 14A). Mice were sacrificed at different time points, after 7 and 28 days from the first NMU exposure. Western blotting analyses showed that the active form of caspase-8 (22 kDa) was solely present in the lung of tumor-bearing mice at both 7 and 28 days post NMU administration, compared to naïve, non-treated mice (Figure 13A and 13B). Taken together, these results appear in contrast in that Caspase-8 is in its active form in both human lung cancer tissues and in murine lung tumor lesions, whereas human NLC tissues, that are ‘healthy’,

absent of macroscopically and clinically detectable tumor lesions, still had caspase-8 in its active form.

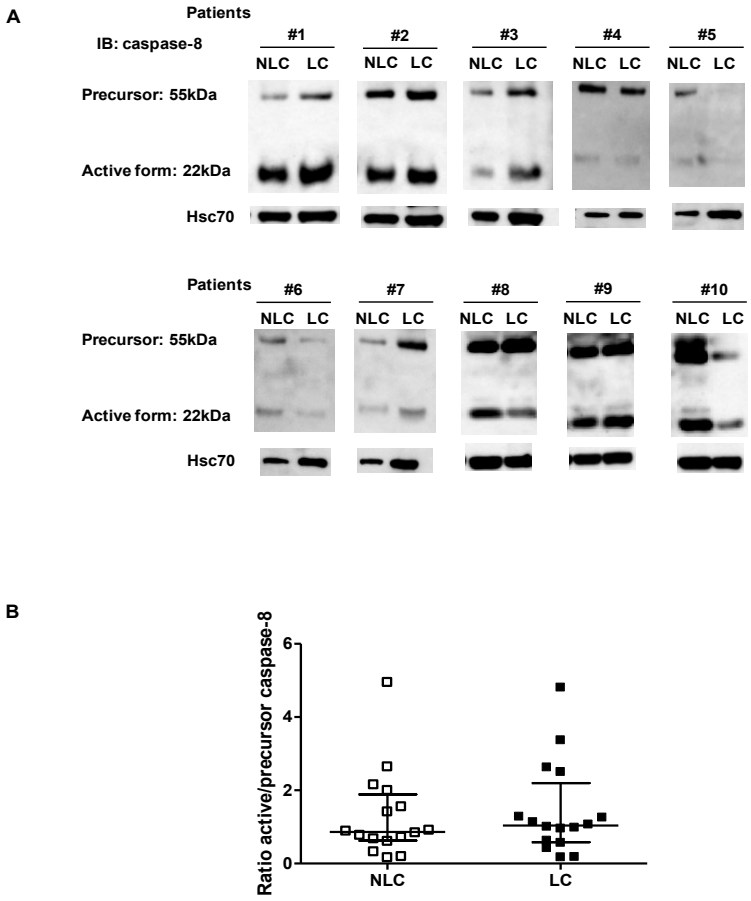

Figure 12. Caspase-8 was active in human samples of NSCLC patients. A) Representative western blotting analyses performed on human samples of NSCLC. Non lung cancer (NLC) and lung cancer (LC) samples had similar levels of the active form of caspase-8 (22 kDa) **(B)**. Data were expressed as ratio of the active (22kDa) vs precursor (55 kDa) form of caspase-8, analyzed by means of Image J Software (NIH, USA). Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

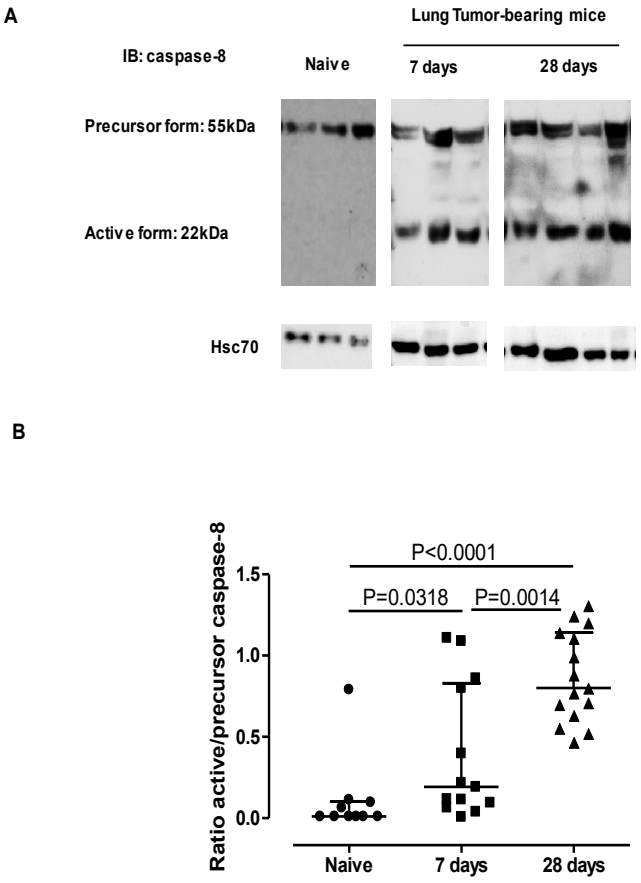

Figure 13. Caspase-8 was active in the lung of NMU-exposed mice. A) Western blot analyses performed on lung homogenates obtained from tumor-bearing mice. **B)** Quantitative analysis (ratio active vs precursor form of caspase-8) showed higher levels of the active form of caspase-8 in lung tumor-bearing mice. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

2.3.2 Pharmacological inhibition of caspase-8 reduced lung cancer progression in mice.

To evaluate the role of caspase-8 during lung cancer establishment/progression, we used a pharmacological inhibitor of the enzyme, z-IETD-FMK (IE 0.5μg/mouse) (Figure 14A). Surprisingly, the administration of IE robustly reduced lung tumor growth compared to control mice (CTR) (Figure 14B and 14C), demonstrating that caspase-8 was involved in lung tumor progression. In addition, we also performed another set of experiments during which caspase-8 was inhibited only after lung tumor formation at 28 days post the first NMU exposure and mice were sacrificed at 35 days. Again, the administration of IE significantly reduced lung cancer outgrowth compared to control mice (Figure 14D).

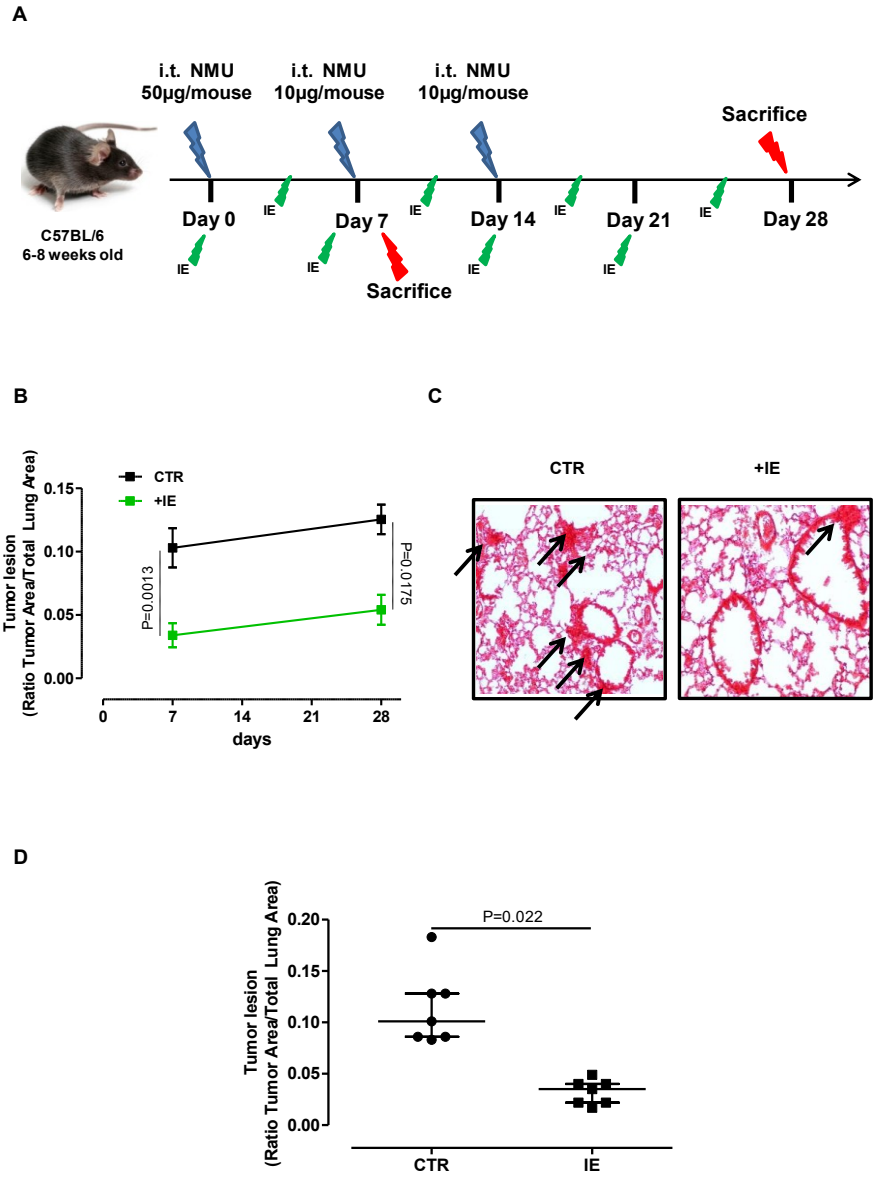

Figure 14. Pharmacological inhibition of caspase-8 reduced lung cancer progression in mice. A) Experimental protocol. **B)** The administration of z-IETD-FMK (IE) significantly reduced lung tumor formation in a time dependent-manner, as shown by H&E pictures in panel **C**. **D)** The inhibition of caspase-8 at 28 days after the first exposure to NMU significantly reduced lung tumor lesions in mice. Experiments were performed in three different experimental days. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

2.3.3 Caspase-8 was involved in pro-inflammatory, but not in cell death, pathways during lung carcinogenesis.

Caspase-8 is an apical caspase which initiates programmed cell death following death receptor ligation[82]. To understand the role of caspase-8 in apoptosis-induced cell death in our mouse model, we went on by analysing the expression of Bcl-2, well-known anti-apoptotic protein[89]. Bcl-2 was highly expressed in the lung of tumor-bearing mice (Figure 15A and 15B) as well as in naïve mice (Figure 15B). However, the pharmacological inhibition of caspase-8 by means of IE did not alter the expression of the anti-apoptotic Bcl-2 in the lung of NMU-exposed mice compared to control mice sacrificed at both 7 and 28 days (Figure 15A and 15B). In contrast, the expression of the short segment of c-FLIP (c-$FLIP_S$ 28 kDa), involved in the apoptotic cell death, was detectable at 28 days in IE-treated tumor-bearing mice compared to the control group (Figure 16A and 16B). Long segment of c-FLIP (c-$FLIP_L$ 51 kDa) was expressed in both control and IE-treated mice (Figure 16A and 16B). Similarly, human tissue of lung cancer (LC) only had the long segment of c-FLIP compared to the short segment, which instead was higher in non cancerous (NLC) lung-derived tissues (Figure 16C), explaining the dichotomy of caspase-8 activation in non-cancerous samples (Figure 12A and 12B).

To note, caspase-8 has also been found to play a number of non-apoptotic roles in cells, such as promoting the activation of the pro-inflammatory NF-κB-dependent signalling[86]. NF-κB controls the transcription of many pro-inflammatory cytokines involved in inflammation-driven carcinogenesis and tumor progression[13]. The administration of IE significantly reduced the release of IL-6 (Figure 17A), TNF-α (Figure 17B), IL-18 (Figure 17C), but not of IL-1β at 28 days (Figure 17D) from the BAL obtained from the lung of NMU-exposed mice compared to the CTR group. In addition, the levels of IL-1α (Figure 17E) and IL-33 (Figure 17F) were significantly reduced in IE-treated

NMU-exposed mice than in CTR lung homogenates obtained from tumor-bearing NMU-exposed mice.

Taken together, our data show that caspase-8 is involved in the amplification of the inflammatory process associated with lung cancer in mice. Instead, its pro-apoptotic activity is strictly related to the presence of the short segment of c-FLIP in both mice and humans.

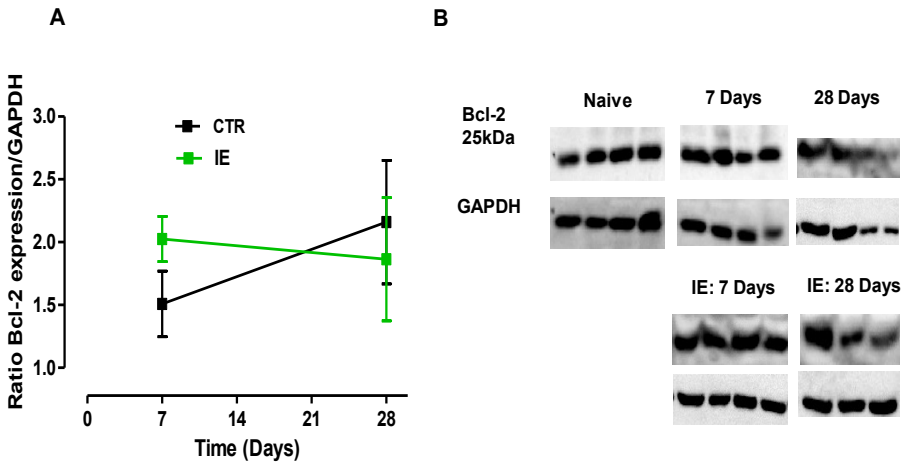

Figure 15. Involvement of Caspase-8 in the apoptotic cascade. Lung tumor-bearing mice presented high levels of Bcl-2 (25 kDa) (**A** and **B**), which were not reduced after IE treatment at both 7 and 28 days. Results are expressed as median±interquartile range (n=7). Experiments were performed in two different experimental days.

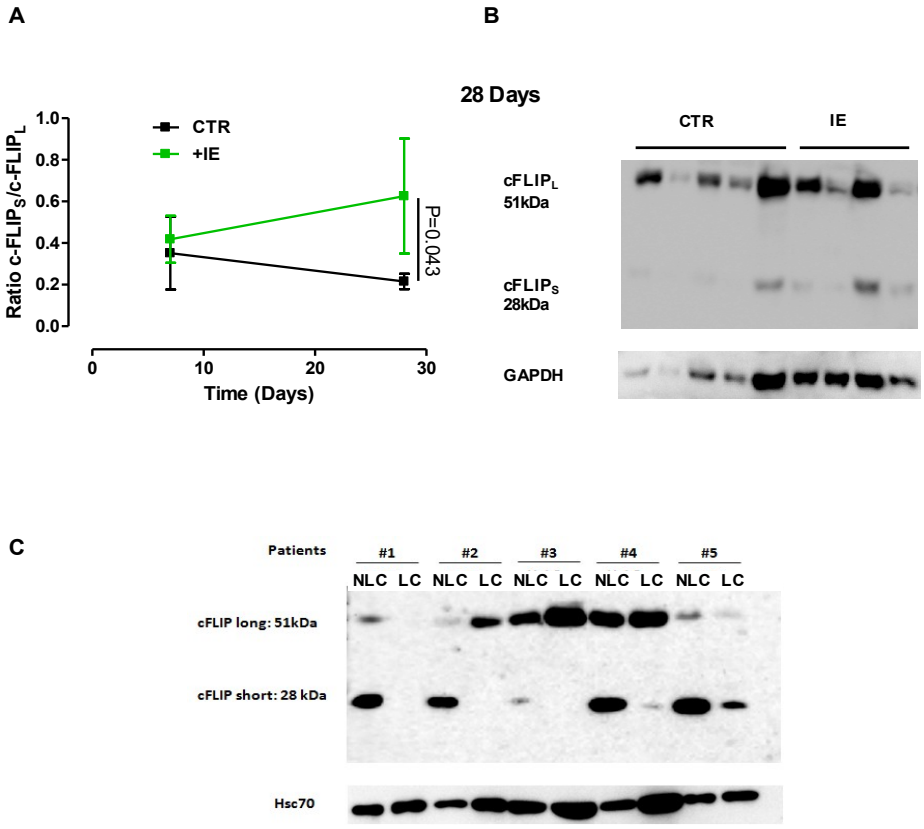

Figure 16. Caspase-8 pro-apoptotic activity was associated with the expression of the short segment of c-FLIP. The levels of the short segment of c-FLIPs (28 kDa) was significantly increased in the lung of NMU-exposed mice treated with IE (**A** and **B**). Western blot analyses were performed on lung homogenates and expressed as ratio between the target protein vs GAPDH or Hsc70 (loading control). **C)** Western blotting analyses showed that human cancerous samples (LC) of NSCLC patients were characterized by the presence of the sole long isoform of c-FLIP (51 kDa) compared to the 'non lung cancer' (NLC) counterpart which instead presented higher levels of the short segment of c-FLIP (28 kDa). Experiments were performed in two different experimental days. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

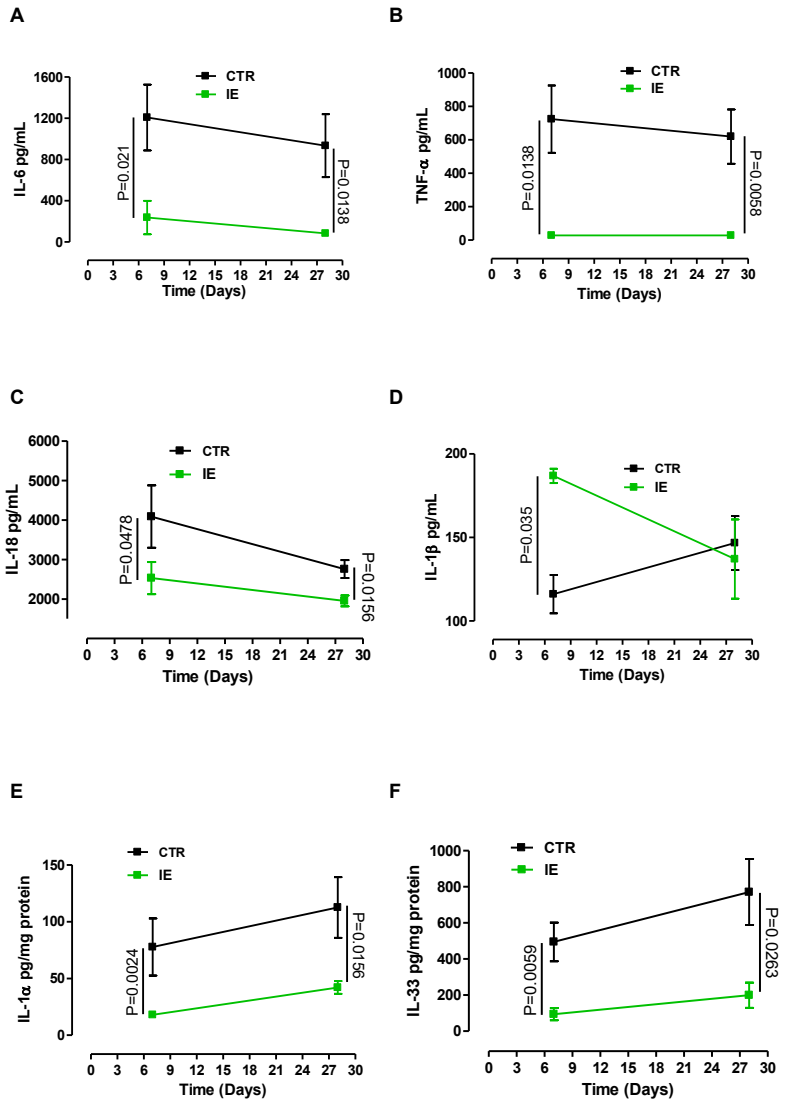

Figure 17. The pharmacological inhibition of caspase-8 reduced pro-inflammatory cytokine release. Broncho-aleveolar lavage (BAL) fluid was harvested from lung tumor-bearing mice at both 7 and 28 days post NMU exposure. The pharmacological inhibition of capase-8 by means of IE significantly reduced the levels of IL-6 (**A**), TNF-α (**B**), IL-18 (**C**) but not of L-1β at 28 days (**D**) in the BAL of lung tumor-bearing mice. Similarly, the levels of IL-1α (**E**) and IL-33 (**F**) were significantly reduced in lung homogenates obtained from control and IE-treated tumor-bearing mice. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

2.3.4 Inhibition of caspase-8 modulated the innate immune microenvironment in the lung of tumor-bearing mice.

Tumor immunoediting has been widely described as pivotal for the success of immunotherapy[90]. Therefore, we moved on by analysing the immune microenvironment in the lung of tumor-bearing mice treated with IE. The inhibition of caspase-8 reduced the percentage of macrophages (identified as $CD11c^{+}CD11b^{+}F4/80^{+}$) in IE-treated tumor-bearing mice compared to control (Figure 18A). Similarly, myeloid-derived suppressor cells (MDSCs: identified as $CD11b^{high}Gr\text{-}1^{high}$) were significantly reduced into the lung of tumor-bearing mice treated with IE compared to control (Figure 18B). In contrast, IE treatment tended to increase the percentage of myeloid DCs (identified as $CD11c^{+}CD11b^{int}F4/80^{-}$) compared to the control group (Figure 18C), although these cells were not in their active form, as we were not able to detect any difference in MHC I/II onto the lung of myeloid DC from control and IE-treated mice (data not shown). Moreover, the percentage of pDCs (identified as $CD11c^{+}B220^{+}PDCA\text{-}1^{+}$) (Figure 18D) and Treg (identified as $CD4^{+}CD25^{+}FoxP3^{+}$) (Figure 18E) did not show any statistical differences between control and IE-treated mice. This latter effect was confirmed by the unaltered levels of IL-10 in the BAL of both control and IE-treated mice compared to naïve mice (Figure 18F).

Taken altogether, these data imply that although the pharmacological inhibition of caspase-8 diminishes the pro-inflammatory humoral pattern of lung tumor microenvironment, it does not interfere with the recruitment of immune-suppressive Treg to the lung of tumor-bearing mice, differently from what observed for caspase-1 (Figure 11B, Chapter 1), implying that its activation does not play a critical role during tumor adaptive immunoediting.

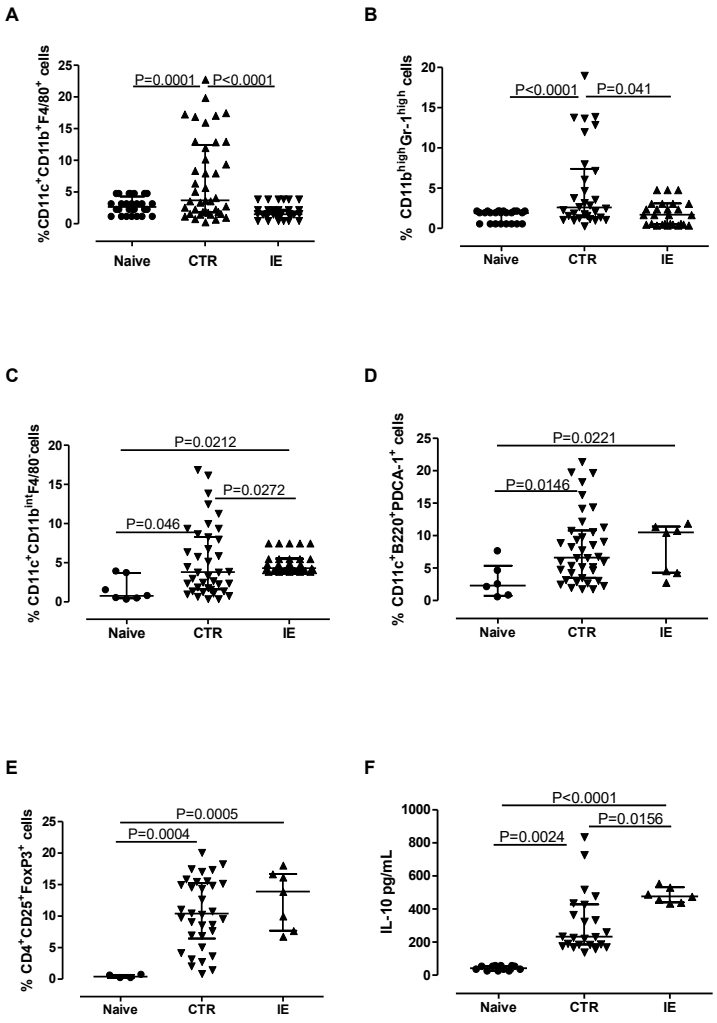

Figure 18. The pharmacological inhibition of caspase-8 modulated the immune microenvironment in the lung of tumor-bearing mice. The pharmacological inhibition of caspase-8 significantly reduced the percentage of macrophages (identified as $CD11c^{+}CD11b^{+}F4/80^{+}$ cells) (**A**), and myeloid-derived suppressor cells (identified as $CD11b^{high}Gr\text{-}1^{high}$ cells) (**B**), but not of dendritic cells (identified as $CD11c^{+}CD11b^{int}$ cells) (**C**), plasmacytoid dendritic cells (identified as $CD11c^{+}B220^{+}PDCA\text{-}1^{+}$ cells) (**D**) and T regulatory cells (identified as $CD4^{+}CD25^{+}FoxP3^{+}$ cells) (**E**). **F)** IL-10 levels in the BAL of control (CTR) and IE-treated lung tumor-bearing mice were not altered, but higher than naïve mice. Experiments were performed at least in two different experimental days. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

2.3.5 Caspase-8 was upstream caspase-1 activation during lung carcinogenesis in NMU-exposed mice.

Data obtained showed that the active form of caspase-8 was involved in lung cancer in both humans and in a mouse model of lung carcinoma. The pharmacological inhibition of the enzyme by means of IE significantly reduced lung cancer outgrowth (Figure 14B and 14C) associated to lower pro-inflammatory response (Figure 17), confirming our main hypothesis about the involvement of the inflammasome in lung cancer. However, as discussed in Chapter 1, even the pharmacological inhibition of caspase-1 significantly reduced lung cancer establishment/progression (Figure 8B), although it did not reduce IL-1β and IL-18 in the BAL of tumor-bearing mice, implying that caspase-1 may act in a non-inflammasome dependent manner or that another orchestrator might govern the effect of caspase-1 in our mouse model of lung cancer.

Concurrently to our study, Gurung P. et colleagues demonstrated that caspase-8 was an upstream regulator of the canonical inflammasome in bone marrow-derived macrophages (BMDMs) and that it was required for caspase-1 activation[91]. Therefore, we compared data for the tumor lesions of y-VAD-treated vs IE-treated mice (Figure 19A) and we found that the inhibition of caspase-1 was associated to a reduction of the tumor lesion less pronounced than caspase-8 inhibition ($P=0.0062$ vs $P<0.0001$).

Therefore to understand the role of both enzymes, we went on by treating NMU-exposed mice with both inhibitors y-VAD plus IE and then compared to the control group and to the group of mice that were solely treated with y-VAD or IE. NMU-exposed-mice were treated with y-VAD (10µg/mouse)+IE (0.5µg/mouse) every two days intraperitoneally (i.p.) after NMU instillation. Animals were sacrificed at 7 and 28 days after the first exposure to NMU. At day 7 tumor size was significantly reduced after the administration of y-VAD+IE, compared to the control mice and to y-VAD-treated mice (Figure

19B). This result was comparable in size to the data obtained from IE-treated mice, confirming the hypothesis that caspase-8 acts upstream of caspase-1. Surprisingly, at a later time point (28 days after the initial NMU instillation), tumor lesion in y-VAD+IE-treated mice was comparable in size to those in CTR mice (Figure 19B).

Therefore, further studies are needed to understand the interplay between these two enzymes during cancer-related inflammatory processes and effective role of the inflammasome activation-associated cell-death pathways.

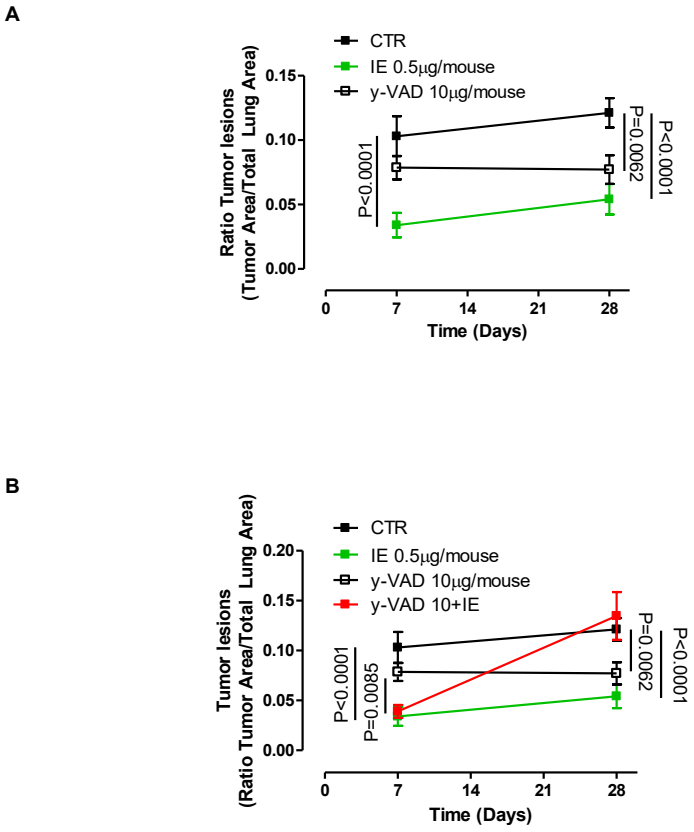

Figure 19. The inhibition of both caspase-1 and caspase-8 did not alter lung tumor progression in mice. A) Comparison of the tumor lesion in CTR, y-VAD- and IE-treated mice. **B)** The inhibition of both caspase-1 and caspase-8 was associated with a tumor lesion comparable in size to those in CTR mice. Data are expressed as medians±interquartile range and analyzed by using Mann Whitney test for non-parametric data.

2.4 Conclusions

The controversial role of caspase-8 in cancer, stimulates the curiosity of numerous scientists. The enzyme has been determined to be highly versatile depending on the cellular context. It has a central role during apoptosis, but can also modulate inflammation propagation, two phenomenon that in an inverse manner, are strictly correlated to tumor outgrowth. Our data showed that pro-caspase-8 was expressed in human NSCLC samples. However, the active form of caspase-8 (22 kDa) was present in both 'healthy, non-cancerous' and cancerous tissues. Similarly, lung tumor-bearing mice had high levels of the active form of caspase-8 after the exposure to the carcinogen. Though, the inhibition of caspase-8 by means of a specific inhibitor, z-IETD-FMK, significantly reduced lung tumor burden accompanied by lower pro-inflammatory cytokine release and innate immune suppressive cells (MDSCs), well-described to facilitate tumor progression[92]. In addition, both mouse and human samples showed that the limiting step for caspase-8 activity in tumor progression was related to the presence of the short segment of c-FLIP. Therefore, caspase-8 is an important orchestrator for cancer-associated inflammation and the presence of the short or long c-FLIP may represent the rheostat for caspase-8-induced tumor proliferation or tumor arrest/regression in the lung.

Alteration of the expression of proteins involved in apoptosis is a common mechanism for tumor cells to evade the pharmacological treatment paving the way for drug resistance[93]. Unlike small cell lung cancer (SCLC), NSCLC, which represents the 80% of lung cancer diagnosis, highly expresses the precursor and the active form of caspase-8 (Figure 12A and 12B). However, the pro-apoptotic activity of caspase-8 depends on the levels of the short segment of c-FLIP[81,82]. Indeed, cancerous tissues of lung cancer in both mice and humans did not express c-FLIPs, instead, the long segment was predominant. When the levels of c-FLIP$_L$ were high, pro-caspase-8

preferentially heterodimerizes with it leading to an insufficient activity of caspase-8 to induce apoptosis, instead favoring cell survival[82]. Therefore, the limiting step for caspase-8-dependent pro-apoptotic activity was related to the presence of the short segment of c-FLIP. Healthy lung tissues had higher levels of c-FLIPs compared to the cancerous tissues, implying that the active form of caspase-8 in the tumor mass was not able to induce apoptosis but rather tumor cell proliferation. Indeed, the inhibition of caspase-8 by means of z-IETD-FMK reduced Ki-67 positive staining in the lung of tumor-bearing mice compared to the control group, implying that caspase-8 can facilitate tumor cell proliferation[66]. In support, the short isoform of c-FLIP was evident only in mice treated with z-IETD-FMK but not in the control group. Lamkanfi and colleagues, reported that the outcome of the pro-apoptotic activity of caspase-8 is most probably determined by the extend of its activation[94]. Our data show that the active form of caspase-8 in both human and mouse samples was not pro-apoptotic, but instead amplified tumor-associated inflammation. The levels of IL-6, TNFα, IL-18, IL-1α, IL-33, but not IL-1β, were significantly reduced in the lung of tumor-bearing mice treated with z-IETD-FMK. The release of these cytokines was associated with STAT-3 activation, a transcription factor able to induce malignant cell proliferation by upregulating the expression of cell cycle regulators and of the anti-apoptotic genes[13]. Indeed, the levels of the anti-apoptotic Bcl-2 were not altered in tumor-bearing mice which were characterized by high levels of the active form of caspase-8. Moreover, we were not able to detect any activation of the effector pro-apoptotic caspase-3, caspase-9 and cytochrome c (data not shown). However, it is to note that Bcl-2 levels were not modulated in the lung of tumor-bearing mice even after z-IETD-FMK treatment, implying that the inhibition of caspase-8 solely alters the inflammatory cytokine pattern without affecting the apoptotic cascade.

Moreover, because NF-kB activation and the amplification of the NF-kB-IL-6/TNF-α-STAT-3 signaling cascade occur in most malignancies and facilitate pro-inflammatory and pro-survival gene expression[95], and because it has been demonstrated that caspase-8 can orchestrate this pathway[75,82,94], we observed that the inhibition of caspase-8 decreased the levels of IKKα, strictly related to NF-kB activation[66]. Therefore, we can speculate that caspase-8-dependent non-canonical inflammasome may represent the rheostat for tumor-associated inflammation. The activation of the inflammasome leads to IL-1β release[13]. In our conditions, we observed that IL-1β release was decreased only at early time points after the inhibition of caspase-8, implying that this cytokine plays redundant activities in our mouse model of lung cancer compared to the other pro-inflammatory cytokines, such as IL-18, IL-1α and IL-33.

High serum concentrations of inflammasome-related IL-1-like cytokines are found in malignancies with low-rate survival from time of diagnosis[18]. These cytokines, directly or via the induction of TNF-α and IL-6[96], are involved in cell proliferation and survival[97], as well as cell adhesion and migration[21], all features of tumor progression and invasiveness. Both pro-IL-1β, pro-IL-18 and pro-IL-33 are converted into their active form mainly by caspase-1-dependent canonical-inflammasome[15]. Although caspase-8 can substitute for caspase-1 and can function as an efficient IL-1-converting enzyme for proteolytic maturation of IL-1β[15], we have not detected significant differences in IL-1β release in the lung of Z-IETD-treated mice and control mice.

Another cytokine associated with the inflammasome complex is IL-1α, an alarmin that unlike IL-1β, IL-18 and IL-33, does not require the cleavage by caspase-1. In models of DEN-induced liver carcinoma[98], skin papillomas[29] and gastric carcinoma[99], IL-1α is released by dying cells, which stimulate oxidative stress pathways, responsible of local inflammation and in some cases of cell rescue from death to provide tissue regeneration and subsequent accumulation of mutations leading to tumor initiation/progression[27]. In support

of these findings, IL-1α-induced IL-6 activates STAT-3 and promotes liver as well as gastric tumorigenesis[21]. Besides its role in tumor-associated inflammation, IL-1α activity was also associated to the activity of mutated K-Ras, one of the main oncogenes, that induces constitutive activation of NF-κB and AP-1, which on one side can promote an autocrine loop for further IL-1α expression/secretion, and on the other increase tumor burden[100]. These processes were well-characterized in a mouse model of pancreatic carcinoma.

To note, the activity of caspase-8 to induce inflammasome in a non-canonical manner has been described in cultured bone-marrow derived cells. Our data is the first, to our knowledge, to demonstrate that the active form of caspase-8 is involved in lung carcinogenesis in vivo in both humans and in a mouse model. In addition, the pharmacological inhibition of caspase-8 proved of anti-tumor activity and represents a novel data not only for the involvement of caspase-8 in lung cancer but also for the involvement of the inflammasome in this context. Though, the presence of the short or long c-FLIP segment may represent the rheostat for caspase-8-induced tumor proliferation or tumor arrest/regression in the lung.

However, the concomitant inhibition of both caspase-1 and caspase-8 in our mouse model of lung cancer does not reveal a reduction of tumor progression at 28 days, compared to the inhibition of inflammation at earlier time points (7 days, Figure 19B). Therefore, these data together the data show in Figure 8B in Chapter 1, implied that caspase-1 and caspase-8 are effector proteases governed by an upstream enzyme that is relevant for lung carcinogenesis.

CHAPTER 3

Caspase-11-dependent non-canonical inflammasome was responsible of lung cancer establishment and progression.

3.1 Introduction

In the face of an ever-expanding bulk of literature on the role of caspase-11-dependent non-canonical inflammasome during cell death pathways, very little is known about its role in cancer and particularly in cancer-associated inflammatory processes.

It is widely described that caspase-11 contributes critically to host defense against bacterial pathogen by triggering pyroptotic cell death of infected myeloid cells[55,101]. Lamkanfi and colleagues suggested that LPS-induced mortality was predominantly due to caspase-11-dependent pyroptotic release of high mobility group box 1 (HMGB1) and potentially other sepsis mediators[48].

Although, the release of "alarmins" such as IL-1α and HMGB1, have been detected in lung cancer tissues and were classified as negative prognostic biomarkers, no evidence correlated caspase-11 to cancer development.

Furthermore, it seemed that caspase-11-dependent pyroptotic cell death occurred independently of the known inflammasome mediators NLRC4, NLRP3 and ASC[17]. In line with that, pro-inflammatory caspase-11 triggered caspase-1-independent macrophages death but, in response to a subset of inflammasome activators, it induced a caspase-1-dependent IL-1β and IL-18 release, resulting in inflammatory response amplification. The interplay between caspase-11, caspase-1 and caspase-8 in cancer is still under-investigated, but it is felt that a more thorough investigation of these pathways in carcinogenesis may help reconcile some of the discrepancies in the field.

3.2 Materials and methods

Mouse model. Female C57Bl/6, Caspase-11 ko (kindly provided by Genentech, USA), caspase-1/11 double ko (DKO), C3H/HeJ (C3H) mice (Charles River, Lecco Italy), were used (6-8 weeks old). Animals were subjected as described in Chapter 1, paragraph 1.2. Briefly, mice were exposed to NMU once a week for three consecutive weeks starting from a high dose of 50μg/mouse (in 10 μl of saline*)* at week 1 followed by other two administrations of 10μg/mouse at week 2 and 3. Animals were sacrificed at different time points (7, 28 and 112 days) to study the involvement of caspase-11 during lung cancer development and progression. Left lung lobes were embedded into OCT medium to perform H&E staining to evaluate the area of the tumor lesion, expressed as ratio of Tumor Lesion Area/Total Lung Area, calculated by means of ImageJ software (NIH, USA). Right lung lobes were digested with 1U/mL collagenase to perform flow cytometry analysis and to measure the release of pro-inflammatory cytokines (IL-1α, IL-1β, IL-18 and IL-33).

Broncho-alveolar fluid (BAL) was collected by means of airway lavage by using a solution of PBS (0.5 mL) and EDTA (0.5 mM) to measure pro-inflammatory (IL-1β and IL-18) cytokine levels.

Isolation of alveolar macrophages. BAL-derived cells were cultured with RPMI supplemented with 10% FBS, L-Glutamine (2mM), penicillin (100U/ml) and streptomycin (100μg/ml) in an atmosphere of 5% CO2 at 37°C. Cells were plated for 1 hour to allow macrophages to attach before removing fluctuant cells. Macrophages purity was checked by means of flow cytometry ($CD11c^+CD11b^+F4/80^+CD169^+$) and was around 85%. Cells were treated for 5 hours with LPS (0.1μg/mL) and/or Glybenclamide (1μM).

Bone-marrow (BM) transplant. Bone-marrow (BM) transplant experiments have been performed using wild type (wt, C57Bl/6 mice) and caspase-11 ko mice. BM-derived cells were isolated from euthanized mice. 6-8 weeks old

recipient mice were irradiated with one dose of 10 x rad to eliminate endogenous BM stem cells and most of the BM-derived cells, before the transplantation of $1x10^6$ donor BM cells, injected into the tail vein of recipient irradiated mice. Animals were divided in four groups: 1. donor wt cells into recipient wt mice (wt into wt); 2. donor caspase-11 ko cells into recipient caspase-11 ko mice (ko into ko); 3. donor wt cells into recipient caspase-11 ko mice (wt into ko); 4. donor caspase-11 ko cells into recipient wt mice (ko into wt).

The degree of chimerism was assessed by FACS analysis of $CD45^+$ blood leucocytes 7-8 weeks after BM transplant. NMU or vehicle were instilled starting at 8 weeks post BM transplant and chimera mice were sacrificed 28 days after the first NMU exposure (Figure 23A).

Cytokine measurements. IL-1α, IL-1β, IL-18 and IL-33 were measured in lung homogenates (expressed as pg/mg of proteins) and in BAL (expressed as pg/mL) by using commercially available ELISAs (eBioscience, CA, USA; R&D Systems, London, UK).

Western blot analyses. Lung homogenates were used to examine the expression of caspase-11 active form (22kDa) vs the precursor form (55 kDa) (Santa Cruz Technologies, CA, USA).

Statistical analysis. Results are expressed as median±interquartile range and analysed with Mann Whitney test for non parametric data. P values less than 0.05 were considered as significant.

3.3 Results

3.3.1 Caspase-11 was responsible of lung cancer progression in mice.

In our previous data, we showed that the pharmacological inhibition of caspase-1 and caspase-8 reduced lung tumor lesions in NMU-exposed mice. This effect was more pronounced for mice where caspase-8 was inhibited. However, when both enzymes were pharmacologically inhibited we did not observe any reduction of tumor formation and progression, implying that the two enzymes are not pivotal for lung carcinogenesis. Moreover, double ko mice (Figure 9, Chapter 1) showed that the genetic absence of both caspase-1 and caspase-11 induced a robustly reduced incidence of lung tumor in NMU-exposed mice. Therefore, to define the role of caspase-11 during lung cancer establishment and progression, C57Bl/6 (CTR) and caspase-11 ko mice (solely ko for caspase-11 and not caspase-1) were treated with NMU and sacrificed at different time points. At 28 days and later time points (112 days) after NMU instillation, the genetic absence of caspase-11 significantly reduced tumor lesion area compared to CTR mice (Figure 20A). More importantly, we observed that at later time points (112 days), the tumor lesion was still low compared to CTR mice, implying that caspase-11 plays a pivotal role for lung carcinogenesis. Our previous data showed that the pharmacological inhibition of the caspase-8 and caspase-1, the latter is the final effector of the inflammasome activation, reduced lung cancer outgrowth associated to lower pro-inflammatory response and to a reduced lung recruitment of immunosuppressive cells. To investigate the role of caspase-11 and the possible co-operation with caspase-1, caspase-1/11 double ko (DKO) and caspase-11 ko NMU-exposed mice were compared in terms of tumor lesion.

Surprisingly, at 28 days after the initial NMU instillation we did not observe any difference in terms of tumor lesion, between caspase-1/11 DKO e caspase-11 ko (Figure 20B) implying that caspase-11, rather than caspase-1, is pivotal for the inflammasome-dependent lung cancer progression and that the enzyme

is upstream caspase-1 to induce the amplification of the occurring inflammatory process associated to lung cancer development, as demonstrated from experiments performed by pharmacologically inhibiting caspase-1 vs DKO (Figure 8B and 9, Chapter 1).

Recent evidence suggested the involvement of TLR4 signaling in regulating caspase-11 expression and as the first signal for inflammasome activation in macrophages infected by pathogens[101]. To investigate whether this pathway/s was involved in our experimental model, C3H mice, which present a polymorphism on TLR4 gene in that the receptor lacks functional activity[102], were used. NMU-exposed C3H mice did not report significant difference in terms of tumor lesion compared to CTR mice at 28 days, implying that TLR4 signalling was dispensable for lung carcinogenesis (Figure 20C). These data are of particular interest in that caspase-11 was reported as the intracellular receptor for LPS after TLR4[103]. In our experimental data, the absence of activity of TLR4 in NMU-exposed C3H mice further confirmed the implication of caspase-11 in lung carcinogenesis.

Taken together, these results reveal that caspase-11 is responsible for lung carcinogenesis in mice and that its activation (Figure 21A and 21B) leads to the canonical and non-canonical activation of the inflammasome during lung cancer establishment and progression.

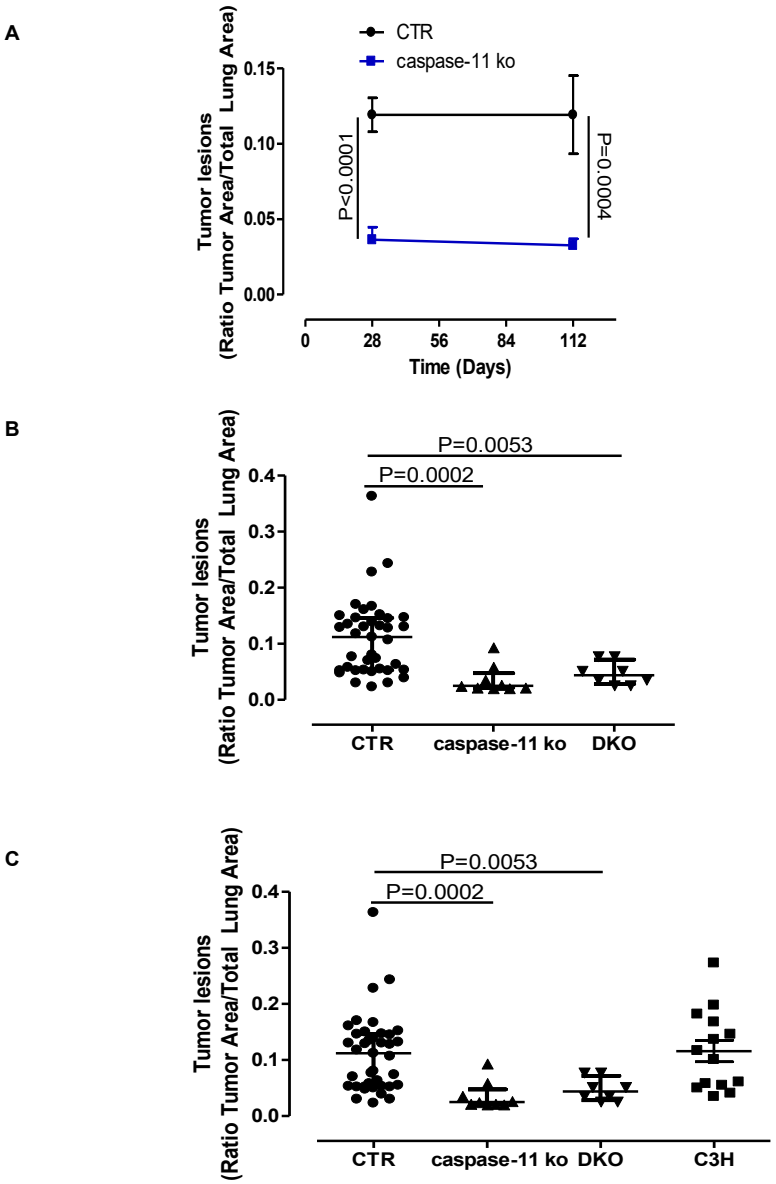

Figure 20. Caspase-11 promoted cancer in the lung mice exposed to carcinogen. A) Caspase-11 ko mice showed a sharp reduction of tumor lesion compared to control mice (CTR) both at 28 and 112 days from the carcinogen instillation. After 28 days from the first exposure to NMU, the lung tumor lesion was similar in caspase-11 ko and caspase-1/11 double ko (DKO) (**B**) and in C3H mice compared to the CTR mice (**C**). Data are expressed as median±interquartile range and analyzed by means of Mann Whitney test for non-parametric data.

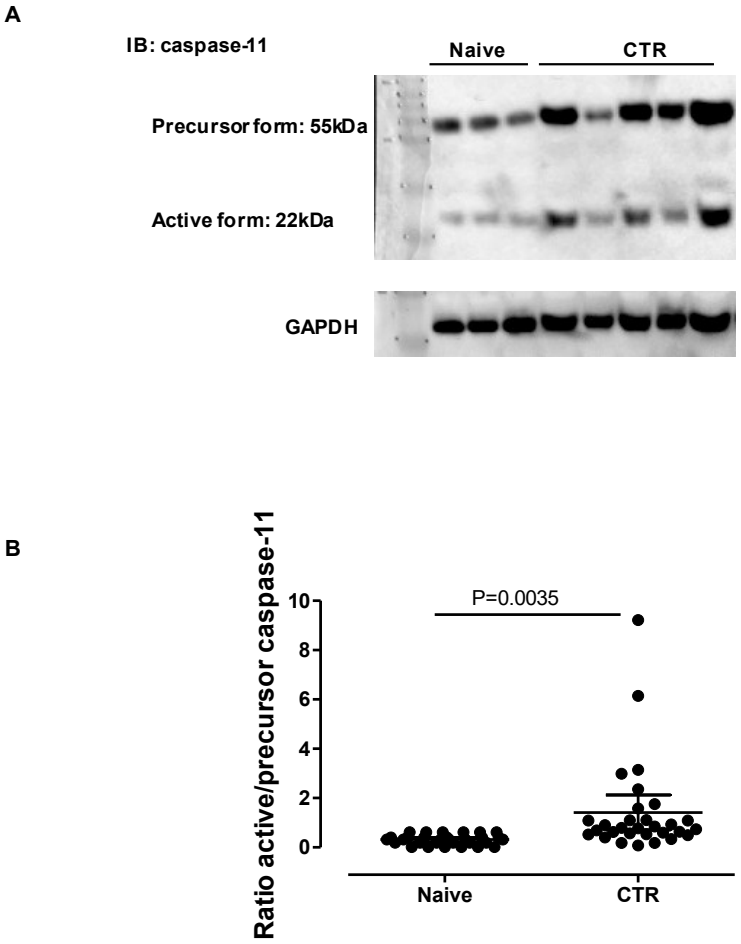

Figure 21. Caspase-11 expression in naïve (non-treated) mice versus NMU-exposed mice (CTR), sacrificed at 28 days from the first NMU exposure. A) Representative western blots. **B)** Quantitative analysis performed by means of Image J (USA, NIH). Data are expressed as median±interquartile range and analyzed by means of Mann Whitney test for non-parametric data.

3.3.2 Caspase-11 modulated the inflammasome activation-dependent pro-inflammatory pathway.

Because the activation of the inflammasome leads to the release of IL-1-like cytokines[13], in order to understand whether the activation of caspase-11 in our experimental model could lead to the release of these cytokines, we analyzed the levels of IL-1-like cytokines in both BAL and lung homogenates. NMU-exposed caspase-11 ko, C3H and DKO mice had significantly reduced levels of IL-18 (Figure 22A); IL-1α (Figure 22B) and IL-33 (Figure 22C). Instead, IL-1β levels were solely reduced in DKO mice (Figure 22D), implying that this cytokine is strictly dependent on caspase-1 rather than caspase-11, although it works upstream caspase-1. Moreover, it is to note that C3H mice still had lower IL-18, IL-1α and IL-33 (Figure 22A, 22B and 22C), implying that although TLR4 is not involved in lung carcinogenesis, it can influence the pro-inflammatory pathway in lung tumor microenvironment.

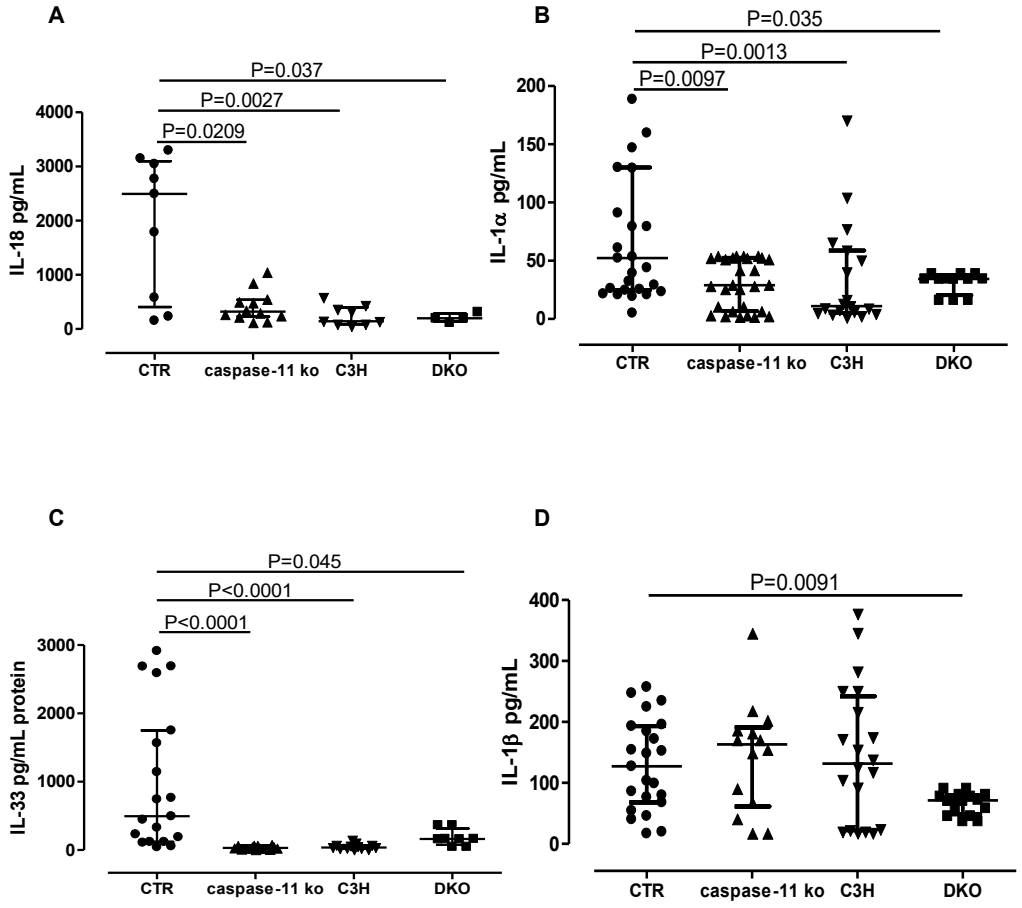

Figure 22. The absence of caspase-11 was associated to a reduced release of pro-inflammatory cytokines from BAL and homogenates of NMU-exposed mice. Caspase-11 ko mice released less IL-18 (**A**) in the BAL, but not IL-1β (**D**) compared to control. Similarly, the levels of IL-1α (**B**) and IL-33 (**C**) were reduced in lung homogenates obtained from caspase-11 ko mice. Data are expressed as median±interquartile range. Significant differences were calculated by means of Mann Whitney test for non-parametric data.

3.3.3 Caspase-11 in lung epithelial cells contributed to lung tumor progression by inducing IL-1α release from macrophages.

The immune system is pivotal for malignant disease control in that failing immunity has been acknowledged for its contribution to cancer development and progression[21]. Tumor microenvironment is a complex scenario in which immune cells play a critical role because they can interfere with tumor cell survival or death[21].

Canonical and non-canonical inflammasome activation has been described in both stromal and hematopoietic cells, although divergent function of the complex are reported depending on the experimental model and on the cell type. Because lung cancer originates from the malignant transformation occurred in the stromal, structural epithelial cells under carcinogen exposure, we wanted to evaluate what cell lineage, structural or hematopoietic, was majorly involved in caspase-11-induced lung cancer establishment and progression.

Therefore, we performed bone-marrow (BM) transplant experiments by using wild type (wt, C57Bl/6 mice) and caspase-11 ko mice, as discussed in the materials and methods section of this Chapter. Animals were divided in four groups: 1. donor wt cells into recipient wt mice (wt into wt); 2. donor caspase-11 ko cells into recipient caspase-11 ko mice (ko into ko); 3. donor wt cells into recipient caspase-11 ko mice (wt into ko); 4. donor caspase-11 ko cells into recipient wt mice (ko into wt). NMU or vehicle were instilled starting at 8 weeks post BM transplant and chimera mice were sacrificed 28 days after the first NMU exposure (Figure 23A). Similarly to CTR and caspase-11 ko mice exposed to NMU, wt into wt and ko into ko group of mice showed similar levels of lung tumor lesion (Figure 23B). More importantly, the tumor lesion was significantly reduced in wt into ko group of mice that presented a wild type hematopoietic lineage and a caspase-11 ko structural (epithelial) lineage, compared to CTR group (Figure 23B). The tumor lesion area was comparable

to that of caspase-11 ko mice (Figure 23B). In contrast, the group of mice that received caspase-11 ko hematopoietic cells but presented a wild type recipient (structural cells) (ko into wt) still presented a significantly reduced lung tumor lesion compared to CTR; however, it was significantly higher than the group of wt into ko mice (Figure 23B). These data highlight the predominant role of caspase-11 in structural epithelial cells where it drives towards lung cancer establishment and progression, confirming our previous hypothesis.

To better understand the role of epithelial vs hematopoietic cells in caspase-11 activation-associated lung cancer, we moved on by analyzing the tumor immune microenvironment by focusing on macrophages because they are widely reported as populating tumor mass and as involved in tumorigenesis[77]. As shown in Figure 24A, the percentage of macrophages recruitment in the lung was reduced in C3H and DKO mice, but surprisingly it was similar between caspase-11 ko and CTR mice. Therefore, to better understand the role of caspase-11-dependent inflammasome in lung tumor-associated macrophages, we isolated and cultured alveolar macrophages from tumor-bearing mice. Macrophages were stimulated for 5 hours with LPS (0.1µg/mL), a TLR4 ligand and well-known inflammasome activator[13]. The stimulation of CTR-derived alveolar macrophages with LPS induced a significant release of IL-1α compared to naïve, non-treated mice (Figure 24B). To verify the involvement of the inflammasome, cells were treated with a NLRP3 inflammasome inhibitor glybenclamide (Gly, 1µM). IL-1α was significantly reduced in LPS+Gly-treated tumor lung macrophages (Figure 24C). Similarly, DKO mice showed that the genetic absence of both caspase-1 and caspase-11 completely abrogated IL-1α release in LPS-treated tumor alveolar macrophages, implying a cross-talk between caspase-1 and caspase-11 (Figure 24D). However, we observed that caspase-11 ko alveolar macrophages released significantly reduced levels of IL-1α when LPS was added (Figure 24E), although caspase-1 was still genetically intact, implying that caspase-11

governs over caspase-1 for the release of IL-1α from lung tumor associated macrophages.

Taken together, these data demonstrate that tumor alveolar macrophages are able to release higher levels of IL-1α than macrophages derived by the lung of naïve mice, implying that the release of IL-1α by macrophages could favor lung carcinogenesis. Furthermore, tumor lung macrophages stimulated by LPS induced IL-1α release in a caspase-11-dependent manner contributing to tumor proliferation.

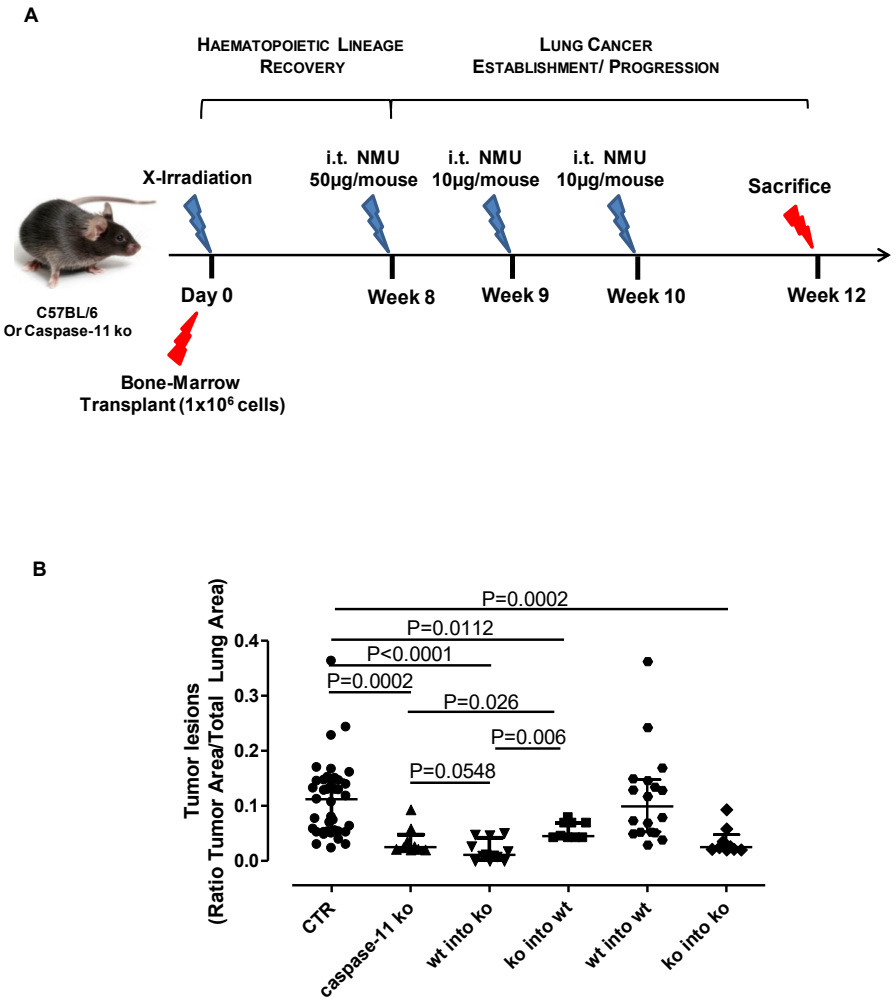

Figure 23. Role of epithelial vs hematopoietic cells in caspase-11-mediated lung cancer establishment/progression. A) Bone-Marrow (BM) transplantation experimental protocol. The tumor lesion was reduced in wt into ko group, while it was increased in ko into wt group compared to caspase-11 ko (**B**). Data are expressed as median±interquartile range. Statistical differences were calculated by using Mann Whitney test for non-parametric data.

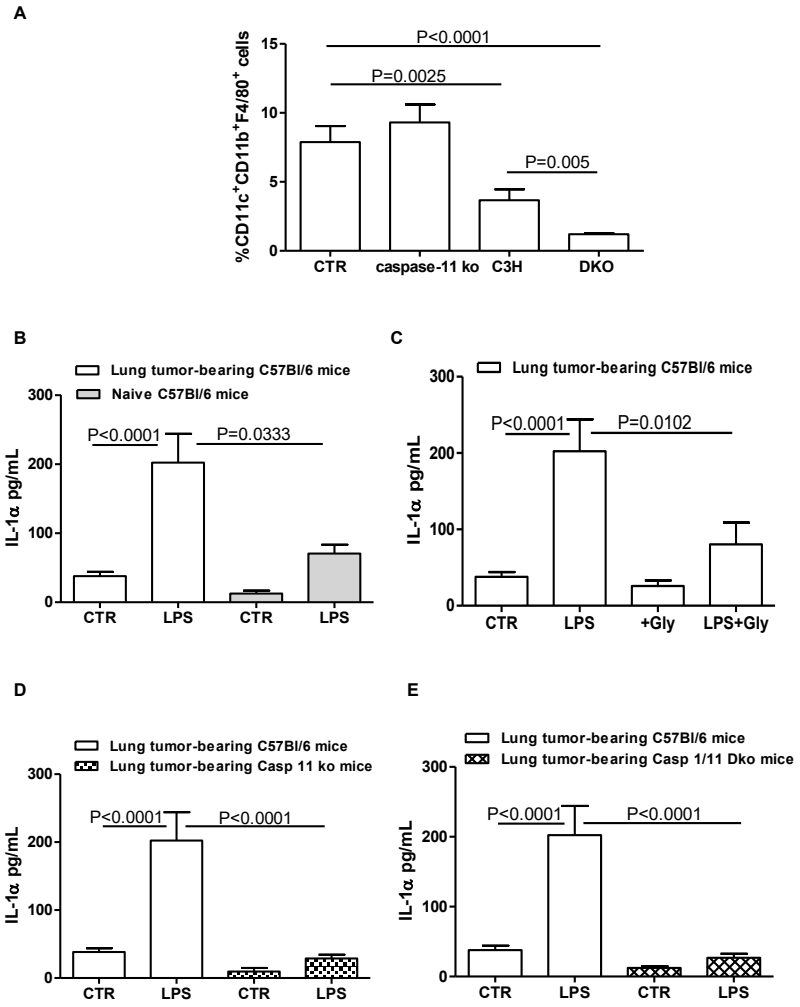

Figure 24. Caspase-11 contributed to lung tumor progression by inducing IL-1α release from macrophages. A) The percentage of macrophages (identified as $CD11c^{+}CD11b^{+}F4/80^{+}$ cells) recruited to the of NMU-exposed C57Bl/6, caspase-11 ko, C3H and DKO mice. Lung tumor-associated macrophages released higher levels of IL-1α after LPS treatment compared to naïve lung-derived macrophages (**B**). The addition of LPS to glybenclamide-treated lung tumor-associated macrophages significantly reduced IL-1α levels compared to cells treated with the sole LPS (**C**). The genetic absence of both caspase-1 and caspase-11 in DKO-derived macrophages obtained from NMU-exposed mice completely abrogated the release of IL-1α after LPS stimulation (**D**). Macrophages obtained from NMU-exposed caspase-11 ko mice released significantly reduced levels of IL-1α when LPS was added (**E**). Data are expressed as median±interquartile range. Statistical differences were analyzed by means of Mann Whitney test for non-parametric data.

3.4 Conclusions

Here, we show that caspase-11 is involved in lung tumor formation associated with the amplification of inflammasome-dependent pro-inflammatory pathways. These data are of novel impact especially if we focus the attention on what reported in literature about the involvement of caspase-11 to induce Gasdermin-mediated cell death. In sharp contrast, we showed that the active form of caspase-11 was associated to lung tumor area. In support, Dr. Hornung's laboratory showed that the activation of the human homologous of caspase-11 did not lead human monocytes to death, as demonstrated on murine bone marrow-derived macrophages. Here we want to pinpoint at the fact that the data reported in literature about the involvement of caspase-11 in cell death is mainly provided by using bone marrow-derived immortalized cells rather than primary and lung cancer-associated immune cells.

In Chapter 1 and 2 we prove that both caspase-8 and caspase-1 are in their active form in human samples of lung cancer and in the lung of an experimental mouse model of lung cancer. However, their involvement to the inflammasome activation in lung cancer was related to an upstream orchestrator that we proved to be caspase-11. Caspase-11 ko mice showed a significant reduction of lung tumor formation.

Subsequently, contrary to what is known about the protective role of the caspase-11 after damage signals, we demonstrate that the main orchestrator of lung cancer-associated inflammatory pathways is caspase-11-dependent non-canonical inflammasome and that it is upstream caspase-1 to induce the amplification of the occurring inflammatory process associated to lung cancer development. Indeed, we observed that the pro-inflammatory IL-18, IL-1α, IL-33, except for IL-1β, were significantly reduced in NMU-treated caspase-11 ko mice- compared to the control.

Another point to worthily focus on is the involvement of TLR4 in lung carcinogenesis. C3H mice, which lack a functional TLR4[102], displayed a

similar lung tumor burden to control mice, implying that it is not involved in lung pro-carcinogenic pathway. However, our group published that TLR4 activation by means of LPS can lead to lung tumor progression[104]. The discrepancy between these latter study could be due to the experimental mouse model. However, it is to note that Shi and colleagues, reported that the cytosolic receptor for LPS was caspase-11[103]. Thus, the apparent discrepancy could be avoided if we consider the actual experiments presented in this thesis. Indeed, it is plausible that what was observed in the published manuscript was still due to caspase-11 activation, that at that time was not widely described as the cytosolic receptor for LPS. Moreover, we cannot completely exclude the role of TLR4 in this pro-carcinogenic pathway since its functional activity in C3H mice showed reduced release of IL-1-like cytokines (Figure 22A, 22B, 22C and 22D).

In addition, of potential interest is the involvement of caspase-11 in the structural cells that lead to the formation of the tumor area versus the pro-inflammatory role in the hematopoietic lineage. Experiments on conditional ablation of caspase-11 could be of further support of our data, but at the moment we do not display of these mice. Nonetheless, wt into ko mice group showed similar levels of lung tumor area as in case of caspase-11 ko compared to the control mice, implying its fundamental role in the structural lineage that can be influenced by the recruitment of the hematopoietic cells, most likely macrophages, that can collaborate with in a caspase-11-depdendent manner to lung carcinogenesis.

DISCUSSION

Lung cancer is the leading cause of cancer death[1], and it is widely accepted that chronic inflammation is an important factor risk for the development of this disease. Chronic inflammation, an aberrantly prolonged form of a protective host response to a loss of tissue homeostasis[105], is linked to cancer development.

The inflammasome has attracted considerable attention ever since its initial characterization due to its implication in the pathogenesis of several human inflammatory diseases[16]. It plays an important role in cellular repair and regeneration following acute damage, but if the signal persists, chronic or excessive inflammasome activity is likely to enhance chronic inflammation-based diseases, as in case of malignancy.

Despite increasingly widespread understanding of the inflammasome involvement in inflammation-driven cancer, the role of the complex during lung cancer establishment and progression remains a topic of intense investigation, since the information about it are few and conflicting.

In the wake of this wave, our interest was projected to understand/investigate the role of the inflammasome in human lung cancer development taking advantage of a mouse model of carcinogen-induced lung cancer. We found that caspase-1, the primary protease associated to the canonical inflammasome, was in its active form in both human lung cancer samples and in a mouse model of lung carcinoma implying that rather than inducing cell death, it is involved in tumor proliferation. The pharmacological inhibition of the caspase-1 by means of Ac-Y-Vad significantly reduced lung cancer outgrowth associated to lower pro-inflammatory response and to a reduced recruitment of immunosuppressive cells (i.e. MDSCs and Treg) to the lung, implying a critical role for the caspase-1 during tumor immunoediting and tumor-associated inflammation. However, the inhibition of caspase-1 did not reduce IL-1β and IL-18 levels in the BAL of tumor-bearing mice, implying

that caspase-1 may act in a non-inflammasome dependent manner or that it was just involved in IL-1α and IL-33 release, other two IL-1-like cytokines strictly dependent on inflammasome activation, or that another orchestrator might govern the effect of caspase-1 in our mouse model of lung cancer.

Concurrently with our studies, Gurung and colleagues demonstrated that caspase-8-dependent non-canonical inflammasome was an upstream regulator of the caspase-1 in bone-marrow-derived macrophages (BMDMs) and that it was required for caspase-1 priming and activation[91]. Based on this concept, we moved on by analyzing the involvement of caspase-8 in our experimental model and the potential cross-talk between these two enzymes during lung cancer establishment and progression. We found that caspase-8 was in its active form in both humans and mice during lung carcinogenesis and that the pharmacological inhibition of the enzyme significantly reduced lung tumor burden accompanied by lower pro-inflammatory cytokines release in mice, without significantly affecting tumor immunoediting. However, it was demonstrated that the activity of caspase-8 as pro-apoptotic and pro-inflammatory was strictly dependent on c-FLIP that in its long segment can inhibit caspase-8 favoring the pro-inflammatory involvement, whereas the short segment of c-FLIP can lead caspase-8 to pro-apoptotic pattern that ends in cells death[84]. In our human samples we found that the active form of caspase-8, present in both non-cancerous and cancerous tissues, was pro-apoptotic in non-cancerous tissues in that it was associated to the expression of the short segment of c-FLIP, whereas its active form was involved in a pro-tumor activity in lung cancer tissues. The experimental mouse data demonstrated that the pharmacological inhibition of the active form of caspase-8 significantly reduced lung tumor burden. However, the inhibition of both caspase-1 and caspase-8 gave surprising results in that it highlighted the role of another orchestrator in the complex scenario of lung carcinogenesis. Indeed, caspase-11 ko mice showed a lower burden of lung tumor in NMU-

exposed mice, effect that was maintained in a time-dependent manner (Figure 20A).

Based on the presented data, we could formulate two potential hypothesis. The first is that caspase-11 is indispensable for the inflammasome involvement in lung carcinogenesis, and it, upstream caspase-1 and caspase-8, orchestrates the inflammasome-dependent pro-inflammatory and pro-carcinogenic pathways (Figure 25). However, due to the relevance of caspase-11 in lung carcinogenesis, as also demonstrated by specific knockout mice, it is plausible and tempting to speculate that besides the pro-inflammatory/pro-carcinogenic role of caspase-11 upstream caspase-1 and caspase-8, another signaling mechanism strictly dependent on caspase-11, probably in an inflammasome-independent manner, can occur. This hypothesis is supported by the fact that the pharmacological inhibition of caspase-1 and caspase-8 (Figure 19A and 19B), leaves unaltered the activity of caspase-11, which could still induce a pro-carcinogenic stimulus for lung cancer establishment and progression. To further understand, we performed additional experiments (not presented in this thesis) that proved that the active form of caspase-11 and its human homologous can bind p53 and most probably sequester it from its oncosuppressive activity. The latter data need further elucidation but opens new perspectives to understand the biology of caspase-11 in an inflammasome-dependent and –independent manner.

In conclusion, we identify a novel mechanism by which lung-associated inflammation due to the exposure to a genotoxic stimulus, can lead to malignancy.

In this scenario, caspase-11 plays a pivotal role in that it favors both a pro-inflammatory and pro-carcinogenic pattern. This effect was principally associated to the expression of caspase-11 in the structural epithelial cells, but, however, to the expression/activity in the hematopoietic cells lineage. In particular, lung tumor-associated macrophages favored lung tumorigenesis via

the activation of caspase-11-dependent inflammasome due to the release of the pro-tumorigenic IL-1α. Tumor-associated alveolar macrophages were able to release higher levels of IL-1α than macrophages derived by the lung of naïve (non-treated) mice, implying that the release of IL-1α by macrophages could favor lung carcinogenesis. It is well reported the correlation between the levels of IL-1α in human lung cancer tissues and the bad prognosis of patients[76]. Moreover, this effect is also associated to the capability of IL-1α to interfere in K-Ras-dependent signaling.

Despite the satisfactory results we obtained to increase the knowledge on the inflammasome biology in lung tumor microenvironment pointing at this complex as a promising therapeutic strategy against lung cancer, there are still many unanswered questions. Therefore, further studies are needed to understand what is/are the upstream inflammasome regulators, what is the role of pyroptosis in this context and how to demonstrate that this cell death pathway associated to inflammasome activation is pro- or anti-tumor, considering the lack of specific methodologies/tools for *in vivo* studies.

Although our data demonstrate the effective involvement of the caspase-1-, caspase-8- and caspase-11-dependent inflammasome during lung cancer establishment and progression in both mice and humans, the exact interplay between these enzyme in cancer is still doubtful, but it is felt that a more thorough investigation of these pathways in carcinogenesis may help reconcile some of the discrepancies in the field.

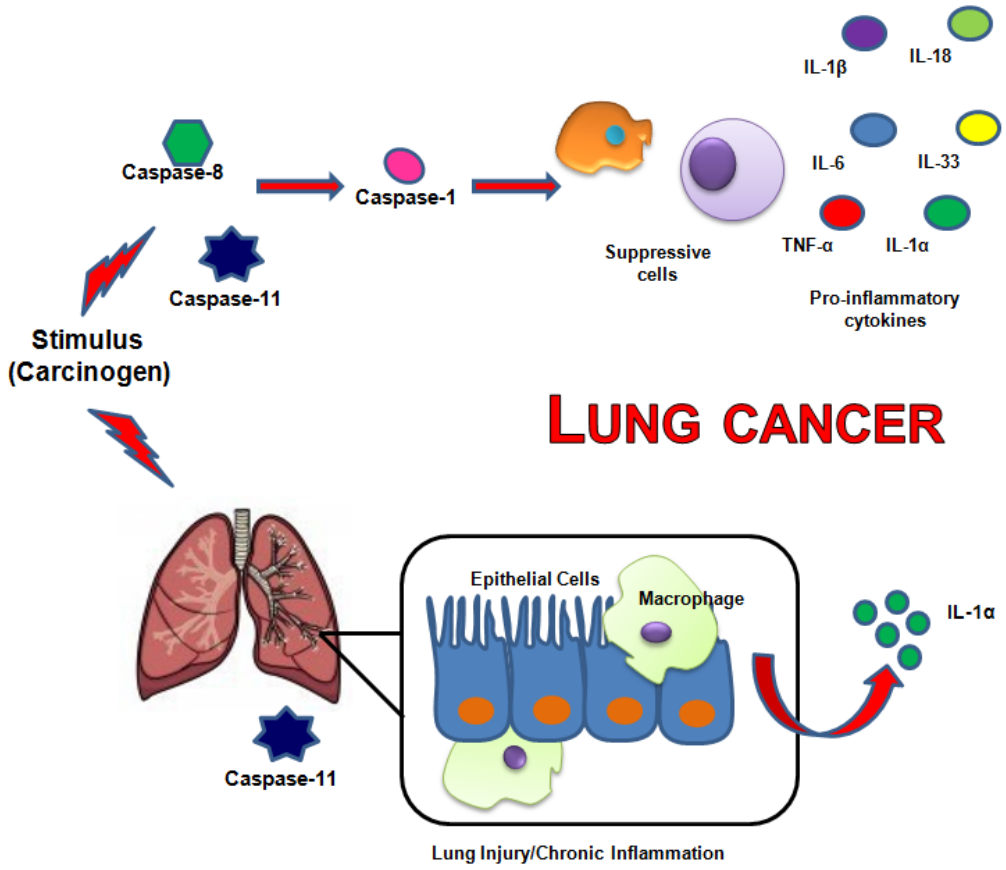

Figure 25. Caspase-11 orchestrates caspase-8- and caspase-1-dependent inflammasome during lung carcinogenesis. In particular, it is involved in both pro-inflammatory and pro-carcinogenisc pathways.

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