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RESEARCH PAPER

Pharmacological inhibition of caspase-8 limits lung tumour outgrowth

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BACKGROUND AND PURPOSE

Lung cancer is one of the leading causes of cancer death worldwide. Despite advances in therapy, conventional therapy is still the main treatment and has a high risk of chemotherapy resistance. Caspase-8 is involved in cell death and is a recognized marker for poor patient prognosis.

EXPERIMENTAL APPROACH

To elucidate the role of caspase-8 in lung carcinoma, we used human samples of non-small cell lung cancer (NSCLC) and a mouse model of carcinogen-induced lung cancer.

KEY RESULTS

Healthy and cancerous NSCLC samples had similar levels of the active form of caspase-8. Similarly, lung tumour-bearing mice had high levels of the active form of caspase-8. Pharmacological inhibition of caspase-8 by z-IETD-FMK robustly reduced tumour outgrowth and this was closely associated with a reduction in the release of pro-inflammatory cytokines, IL-6, TNF-α, IL-18, IL-1α, IL-33, but not IL-1β. Furthermore, inhibition of caspase-8 reduced the recruitment of innate suppressive cells, such as myeloid-derived suppressor cells, but not of regulatory T cells to lungs of tumour-bearing mice. However, despite the well-known role of caspase-8 in cell death, the apoptotic cascade (caspase-3, caspase-9 and Bcl-2 dependent) was not active in lungs of z-IETD-treated tumour-bearing mice, but instead higher levels of the short segment of c-FLIP (c-FLIPs) were detected. Similarly, human healthy lung samples had higher levels of c-FLIPs than cancerous samples.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that caspase-8 is an important orchestrator of cancer-associated inflammation and the presence of short segment of c-FLIP determines whether caspase-8 induces tumour proliferation or tumour arrest/regression in the lung.

Abbreviations

DC, dendritic cell; IE, z-IETD-FMK; MDSC, myeloid-derived suppressor cell; NMU, N-methyl-N-nitrosourea; pDC, plasmacytoid DC

Tables of Links

TARGETS	
Catalytic receptors[a]	**Enzymes**[b]
B220 (RTP type C)	Caspase 3
CD11b (Integrin, α M subunit)	Caspase 4 (11)
CD11c (Integrin, α X subunit)	Caspase 8
CD25 (IL-2α receptor)	Caspase 9
Fas (CD95)	IKKα
TNFR1	p63
	RIP3

LIGANDS	
Fas ligand	IL-18
c-FLIP	IL-33
IL-1α	TNFα
IL-1β	TRAIL
IL-6	z-IETD-FMK
IL-10	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Lung cancer is one of the main causes of cancer death worldwide (Pinto *et al.*, 2011). Although there have been advances in therapy, conventional therapy still remains the main treatment and it has a low success rate and high risk for the development of chemotherapy resistance (Juin *et al.*, 2013). Therefore, novel therapeutic approaches are needed to improve patient prognosis and survival rate, which is still less than 15% from time of diagnosis (Pinto *et al.*, 2011).

One of the main mechanisms of chemotherapy resistance is associated with the evasion of tumour cells from apoptotic, non-inflammatory cell death (Riley *et al.*, 2013). Caspase-3 and caspase-9 represent the main caspase effectors that lead to apoptotic cell death (Morello *et al.*, 2009). However, other caspases are involved in apoptosis-mediated cell death. In fact, caspase-8 is one of the upstream mediators of cell death and its activation is associated with an increase in TNF-like cytokines, such as TNF-α and Fas ligand (FasL; CD95) (Blander, 2014).

Caspase-8 is a cysteinyl protease, which is activated through the formation of a death-inducing signalling complex when death receptors are complexed to their specific ligands. Engagement of death receptors such as Fas (CD95), TNF-related apoptosis-inducing ligand (TRAIL) and TNF receptor 1 (TNFR1) results in the recruitment of caspase-8 and its adaptor Fas-associated protein with death domain (FADD), which initiates an apoptosis cascade that leads to the induction of caspase-3 (extrinsic)-dependent and caspase-9 (intrinsic)-dependent pathways (Blander, 2014). However, although the fine relationship between apoptosis and inflammation has been separated for years, caspase-8 has recently been demonstrated to mediate the priming and activation of the canonical and noncanonical NOD-like receptor, pyrin domain containing 3 (NLRP3) inflammasome (Gurung *et al.*, 2014). The activation of caspase-8 induces inflammasome-dependent IL-1β release from macrophages not only in a canonical, but also in a noncanonical manner by promoting the activation of caspase-1. Indeed, caspase-1/4 knockout macrophages were still able to release IL-1β under infection conditions (Sagulenko *et al.*, 2013). In this context, caspase-8 inhibits the kinase RIP3, which is involved in necrosis and predominates in caspase-8-induced apoptosis (Kaiser *et al.*, 2011; Oberst *et al.*, 2011). Notably, mice deficient in caspase-8 are embryonic lethal, indicating the important role of caspase-8 not only in regulating necrotic cell death, but also at inducing proliferation, as demonstrated in T lymphocytes (Kawadler *et al.*, 2008). The proliferation of activated T lymphocytes that leads to NF-κB and MAPK activation is regulated by caspase-8. Hence, caspase-8 can, therefore, be categorized as both an apoptotic/non-inflammatory caspase and pro-inflammatory caspase together with caspase-1 and caspase-4 (also known as caspase-11).

Caspase-8 is inactivated in a variety of human cancers (Fulda, 2009), which may promote tumour progression as well as resistance to current treatment approaches. Shivapurkar *et al.* (2002) demonstrated that caspase-8 was silenced in small cell lung cancer (SCLC), suggesting that its absence was responsible for tumour cell chemoresistance under TRAIL stimulation. In contrast, Riley *et al.* (2013) demonstrated that pro-caspase-8 is overexpressed in non-small cell lung carcinoma (NSCLC) and represented a marker for poor patient prognosis. Therefore, the aim of our study was to elucidate the role of caspase-8 in human lung carcinoma (NSCLC) taking advantage of a mouse model to better understand its role in tumour outgrowth.

Here, we demonstrated that pro-caspase-8 was expressed in human NSCLC samples. However, the active form of caspase-8 (p22 kDa) was present in both 'healthy' and cancerous tissues. Similarly, lung tumour-bearing mice had high levels of the active form of caspase-8 after exposure to the carcinogen. The inhibition of caspase-8 by means of a specific inhibitor, z-IETD-FMK, significantly reduced lung tumour burden and this was accompanied by the release of lower levels of pro-inflammatory cytokines and innate immune suppressive cells (myeloid-derived suppressor cells, MDSCs), that are well known to facilitate tumour progression (Pinto *et al.*, 2011). In addition, both mouse and human samples showed that the limiting step for caspase-8 activity in tumour progression was related to the presence of the short segment of c-FLIP (c-FLIPs). Therefore, caspase-8 is an important orchestrator of cancer-associated inflammation and the pres-

ence of the short or long c-FLIP may determine whether caspase-8 induces tumour proliferation or tumour arrest/regression in the lung.

Methods

Human samples

We used human samples of non-small cell lung cancer (NSCLC) from patients undergoing thoracic surgery at the Hospital of Salerno 'San Giovanni di Dio e Ruggi D'Aragona' after the approval of the patients, who signed a written informed consent approved by the Review Board of the Ethics Committee of the University Hospital of Salerno. The subjects with NSCLC were 60 ± 10 years of age as described in Table 1. Samples from the tumour mass were defined as 'cancerous' (LC), whereas the 'healthy' (H) samples were obtained from the same patient from a distant non-cancerous portion of the lung. Human samples were collected within 12 h of the thoracic surgery and processed within the following 3 h.

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. 116/92) and European Community Council for Animal Care (Directive 2010/63/EU) on the protection of animals used for scientific purposes and were approved by San Giovanni di Dio e Ruggi D'Aragona-University-Hospital of Salerno Ethical Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Experimental protocol

To investigate the role of caspase-8-dependent non-canonical inflammasome in the establishment/progression of lung carcinoma, a carcinogen-induced mouse model of lung cancer was used. After anaesthesia with isoflurane (100%; the depth of anaesthesia was evaluated by monitoring the consciousness of the mice), N-methyl-N-nitrosourea (NMU), a well-known carcinogen and mutagen due to its alkylating activity (Karran *et al.*, 1979; Lin *et al.*, 2010; Freire *et al.*, 2013), was intratracheally (i.t.) instilled into C57Bl/6 mice (female; 6–8 weeks of age) for 3 consecutive weeks, starting with a high dose of 50 µg per mouse (in 10 µL of saline) at week 1 followed by another two doses of 10 µg per mouse at weeks 2 and 3 (Figure 2A). Mice were divided into the following groups: (1) naïve, non-treated, mice; (2) CTR (control), i.t. instilled with NMU; and (3) lung cancer-bearing mice treated with Z-IETD-FMK (0.5 µg per mouse), a specific caspase-8 inhibitor (Man *et al.*, 2013). To determine the involvement of caspase-8 in lung cancer development the animals were killed at different time points (3, 7 and 28 days). Left lung lobes were embedded in optimal cutting temperature medium and stained with hematoxylin & eosin to evaluate the number of tumour foci/lung, and the phenotype of cells in the tumour lesion; these were then expressed as ratio of hyperplastic cells/lung section or as ratio tumour lesion area/total lung area, calculated with ImageJ software (NIH, USA). Moreover, tumour incidence was analysed as the percentage of tumour formation of each lung compared with the average of all tumour area/total lung area of n = 39 mice treated with NMU.

Table 1

NSCLC patients' details

Patient No.	Gender	Age (years)	Smoker	Type of lung cancer
1	M	70	Yes	Adenocarcinoma
2	M	61	Yes	Adenocarcinoma
3	M	73	Yes	Adenocarcinoma
4	M	53	Yes	Squamous carcinoma
5	M	67	No	Adenocarcinoma
6	M	62	No	Adenocarcinoma
7	M	57	No	Adenocarcinoma
8	M	70	Yes	Squamous carcinoma
9	F	53	No	Adenocarcinoma
10	M	62	Yes	Adenocarcinoma
11	M	73	No	Squamous carcinoma
12	M	59	No	Adenocarcinoma
13	F	63	Yes	Adenocarcinoma
14	F	71	No	Squamous carcinoma
15	M	58	Yes	Adenocarcinoma
16	F	65	No	Adenocarcinoma

Pro-inflammatory (IL-18, IL-33 and TNF-α) and anti-inflammatory (IL-10) cytokine levels were measured in bronchoalveolar fluid (BAL), collected by means of airway lavage (0.5 mL) using a solution of PBS and EDTA (0.5 mM).

Flow cytometry analysis

Lungs were isolated and digested with 1 U·mL^{-1} collagenase (Sigma Aldrich, Rome, Italy). Cell suspensions were passed through 70 μm cell strainers, and red blood cells were lysed. To investigate the immune infiltrates in the lungs of tumour-bearing mice, lung cell suspensions were labelled with specific antibodies (CD11c, CD11b, B220, F4/80, 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^{-1} of proteins) and in BAL (expressed as pg·mg^{-1} of proteins) by using commercially available ELISAs (eBioscience, San Diego, CA, USA; R&D Systems, Abingdon, UK).

Western blot analysis

Lung homogenates were used to examine the expression of caspase-8 active form (22 kDa; Santa Cruz Biotechnologies, Inc., Dallas, TX, USA). Similarly, c-FLIP, Bcl-2 and IKKα (Santa Cruz Technologies) expression was evaluated.

Immunofluorescence analyses

Left lung cryosections were analysed for the presence of Ki-67 (Dako, Cambridge, UK), p63 and cytokeratin 5 (K5) (Santa Cruz technologies). 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: 63×; Carl-Zeiss, Jena, Germany).

Statistical analysis

Results are expressed as means ± SEM. Changes observed in treated groups compared with controls were analysed using Student's *t*-test. *P*-values less than 0.05 were considered significant.

Results

Caspase-8 is active in both humans and mice during lung carcinogenesis

Pro-caspase-8 was reported as overexpressed in the lung of cancer patients (Riley et al., 2013). However, its role is still underinvestigated in NSCLC patients. Surprisingly, both healthy (H) and cancerous (LC) human samples had similar levels of the active form (22 kDa) of caspase-8 compared with the precursor, inactive form (55 kDa) (Figure 1A). Data were also analysed as ratio of precursor or active caspase-8 versus Hsc70. As shown in Figure 1B and C, no statistical differences were registered between H versus LC lung samples.

In order to elucidate the role of caspase-8 in lung carcinogenesis, we used a mouse model of carcinogen-induced lung cancer. C57Bl/6 mice were i.t. injected with NMU once a week for 3 consecutive weeks (Figure 2A). Western blotting analysis showed that the active form of caspase-8 (p22 kDa) was present in the lung of tumour-bearing mice at both 7 and 28 days compared with naïve (non-treated) mice (Figure 2B and C). However, we could not detect statistically significant differences in the active caspase-8 levels between naïve vs. tumour-bearing mice killed at 7 days, although the levels tended to be increased in lung tumour-bearing mice killed at 7 days (Figure 2C), implying that the activity of this enzyme is predominant during cancer development. According to a blinded pathologist, the lungs of NMU-treated mice killed at 7 days showed inflamed lesions, whereas hyperplastic/tumour lesions were visible starting at 4 weeks in the lungs of NMU-treated mice (Figure 2D and E). In our experimental conditions, the incidence of lung tumours in mice was around 80% (Supporting Information Fig. S1A). The tumours in the NMU-treated mice were adenocarcinoma-like according to immunofluorescence analyses (Supporting Information Fig. S1B), which showed that lung tumour cells were Ki-67-, K5 and p63-positive, similar to the human lung adenocarcinoma (Xiao *et al.*, 2013). Pharmacological inhibition of caspase-8 by means of z-IETD-FMK (0.5 μg per mouse) significantly reduced both the number of hyperplastic cells (Figure 2D) and tumour lesions, measured as tumour area (Figure 2E), at 4 weeks. Similarly, the number of tumour foci in lungs of NMU-treated mice at 4 weeks after z-IETD-FMK treatment was reduced compared with CTR mice (Figure 2F and G). In support of this, z-IETD-FMK treatment reduced both Ki-67-positive cells (Figure 2H) and the incidence of lung tumours in NMU-treated mice compared with the control group (Supporting Information Fig. S1A).

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

Because caspase-8 has been widely associated with cell death and has recently been shown to have a pro-inflammatory role, we determined the effects of a pharmacological caspase-8 inhibitor, z-IETD-FMK, which was administered i.p. twice a week. Surprisingly, the administration of z-IETD-FMK ($n = 12$) robustly reduced lung tumour growth compared with control mice ($n = 12$) (Figure 2D and E). In addition, we also performed another set of experiments during which caspase-8 was inhibited only after lung tumour formation at 4 weeks after the first NMU treatment. Again, the administration of z-IETD-FMK significantly reduced lung cancer outgrowth compared with control mice (Supporting Information Fig. S2).

Taken together, these results demonstrate that the activation of caspase-8 is involved in the development/progression of lung tumours.

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

The central role of caspase-8 in apoptotic processes has prompted significant clinical interest in regulating its expression and proteolytic activity (Riley *et al.*, 2013). To understand the role of caspase-8 in apoptosis-induced cell death in our mouse model, we analysed the expression of Bcl-2, a well-known anti-apoptotic protein. Lung homogenates derived from control and z-IETD-FMK-treated mice were used for Western blotting analyses. Bcl-2 expression was highly

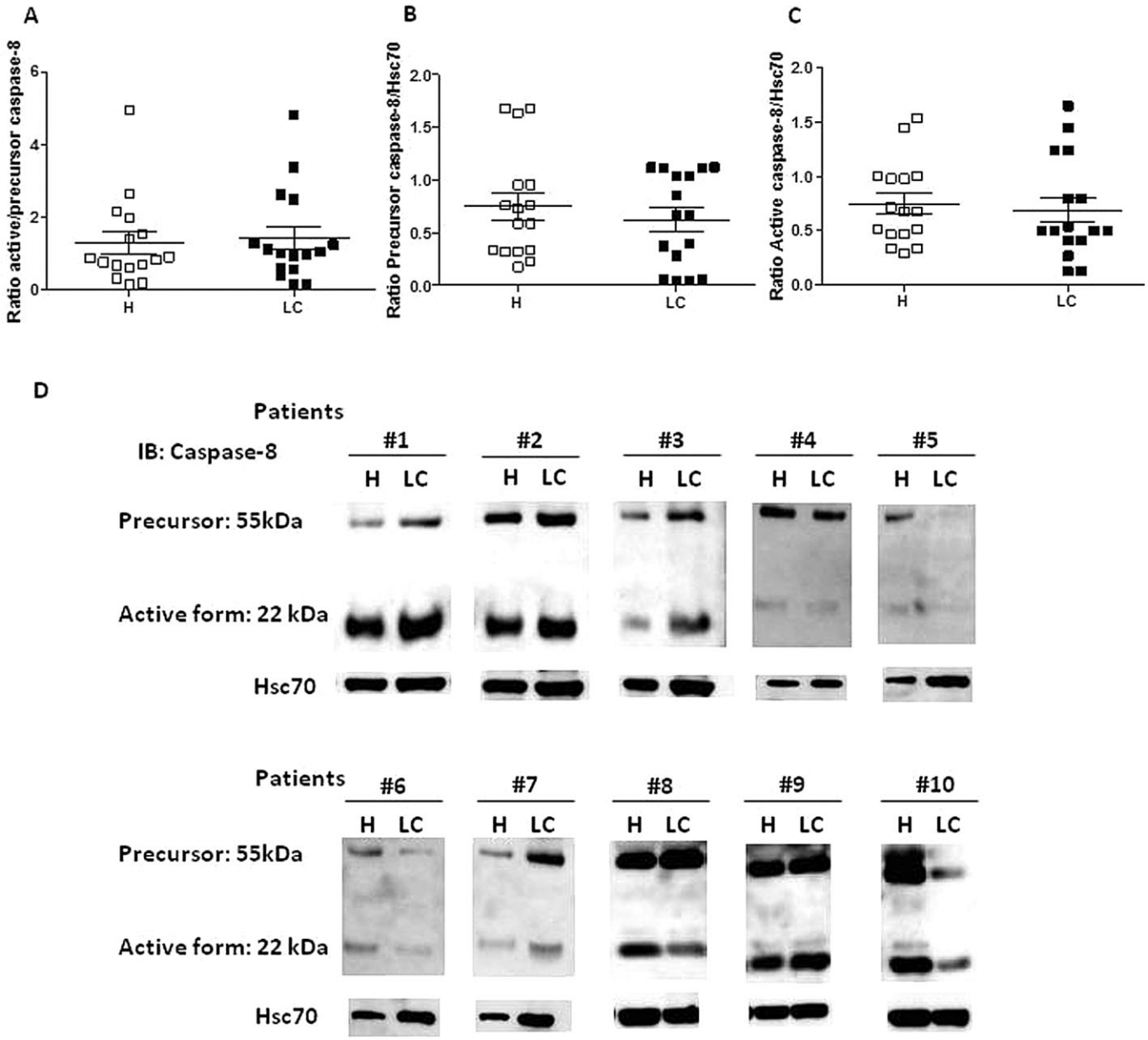
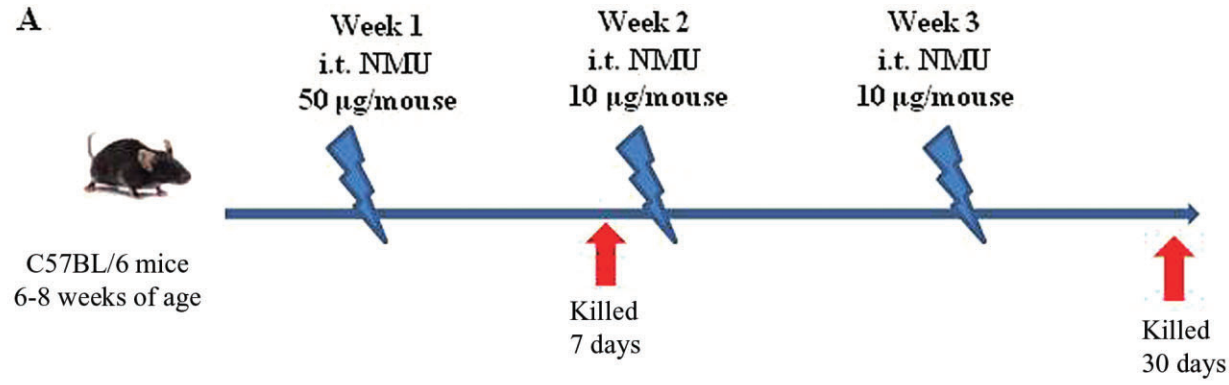


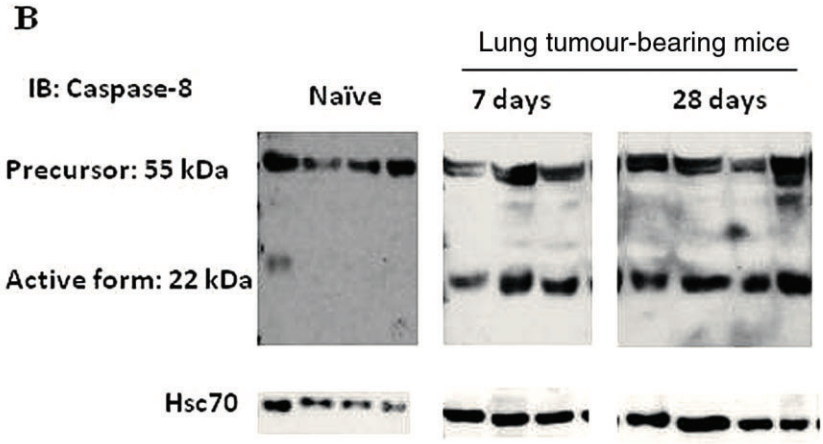
Figure 1

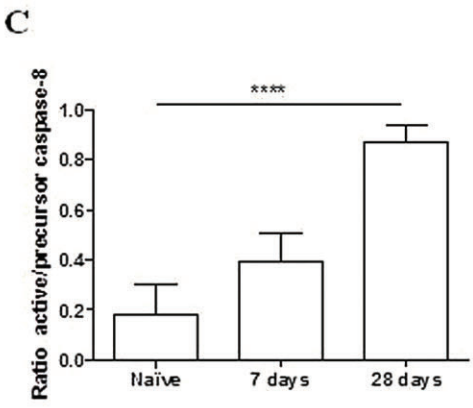
Caspase-8 is active in human samples from NSCLC patients. Human NSCLC samples were enzymatically digested and subjected to Western blotting analyses for caspase-8. (A) We did not detect statistically significant differences between the active form of caspase-8 (p22 kDa) vs. the precursor form of caspase-8 between 'healthy' (H) and 'cancerous' (LC) lung samples. Data were also analysed as ratio of (B) precursor or (C) active caspase-8 versus Hsc70 (loading control). (D) Representative Western blotting pictures. Data were analysed by means of ImageJ software (NIH).

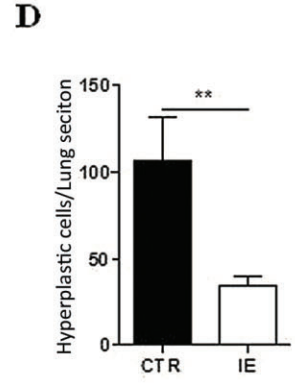
expressed in lung tumour-bearing mice (Figure 3A, $n = 7$) as well as in naïve mice (Figure 3B, $n = 4$). However, z-IETD-FMK did not alter the expression of the pro-apoptotic Bcl-2 in lung tumour-bearing mice compared with control mice ($n = 7$), when killed at either 7 or 28 days (Figure 3A and B). In addition, we were not able to detect the active form of the pro-apoptotic caspase-3 and caspase-9 (data not shown) in either control or z-IETD-FMK-treated lungs of tumour-bearing mice. In contrast, the expression of the short segment c-FLIP (28 kDa), which is involved in apoptotic cell death (Blander, 2014), was detectable at 28 days in z-IETD-FMK-treated tumour-bearing mice but not in the control group (Figure 3C and D). However, the long segment (51 kDa) of c-FLIP was expressed in both control and z-IETD-FMK-treated mice (Figure 3C and D). Similarly, human samples of NSCLC only had the long segment of c-FLIP and not the short segment, which instead was higher in healthy (H) lung-derived samples (Supporting Information Fig. S3).

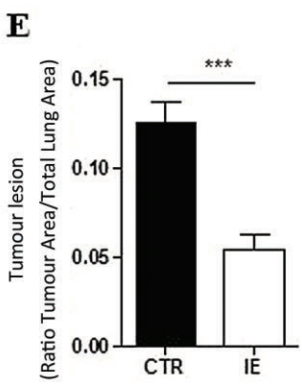
Of note, caspase-8 has also been shown to be involved in a number of non-apoptotic pathways, such as activation of

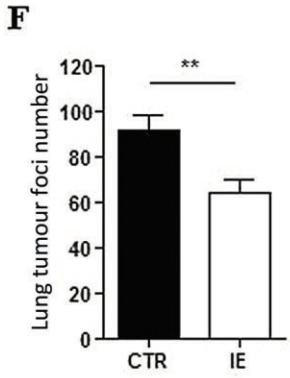

A
Week 1
i.t. NMU
50 µg/mouse
Week 2
i.t. NMU
10 µg/mouse
Week 3
i.t. NMU
10 µg/mouse
C57BL/6 mice
6-8 weeks of age
Killed
7 days
Killed
30 days

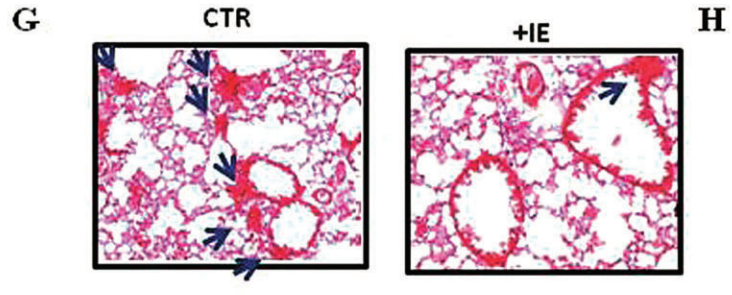

B
Lung tumour-bearing mice
IB: Caspase-8
Naïve
7 days
28 days
Precursor: 55 kDa
Active form: 22 kDa
Hsc70


C
****
Ratio active/precursor caspase-8
1.0
0.8
0.6
0.4
0.2
0.0
Naïve
7 days
28 days


D
**
Hyperplastic cells/Lung seciton
150
100
50
0
CTR
IE


E
***
Tumour lesion
(Ratio Tumour Area/Total Lung Area)
0.15
0.10
0.05
0.00
CTR
IE


F
**
Lung tumour foci number
120
100
80
60
40
20
0
CTR
IE


G
CTR
+IE

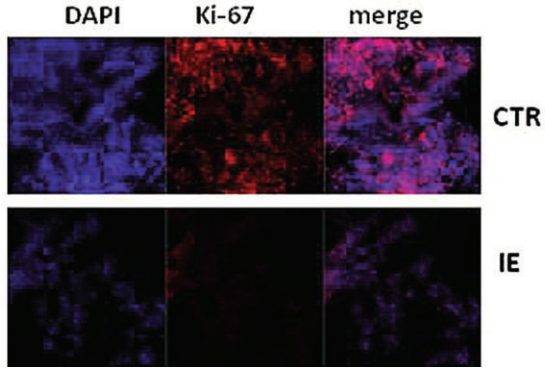

H
DAPI
Ki-67
merge
CTR
IE

Figure 2

Caspase-8 is active in the lung of tumour-bearing mice subjected to carcinogen exposure. (A) Experimental protocol: C57Bl/6 mice (female, 6–8 weeks of age) were treated i.t. with NMU, a well-known mutagen/carcinogen, for 3 consecutive weeks at a dose of 50 μg per mouse at week 1 and at the dose of 10 μg per mouse at weeks 2 and 3. Mice were killed at different time points, 7 and 28 days after the first NMU treatment. (B) Western blot analyses performed on lung homogenates obtained from tumour-bearing mice showed both the precursor (55 kDa) and the active (p22 kDa) form of caspase-8 compared with naïve mice. Ratio between the active and the precursor form of caspase-8 was shown in panel C. In another set of experiments, lung tumour-bearing mice were treated with caspase-8 inhibitor z-IETD-FMK (IE) every 2 days starting from the first NMU treatment. The administration of z-IETD-FMK significantly reduced hyperplastic cells (D), lung tumour formation (E) and lung tumour foci number (F), as shown by hemtoxylin & eosin staining in panel G. (H) Immunofluorescence analyses of Ki-67-positive cells (Alexa-Fluor-555) (magnification: 63×). Hyperplastic cells were counted in various lungs section and expressed as hyperplastic cells per lung section, whereas tumour lesions are expressed as ratio between tumour area versus total lung area. Data represent mean ± SEM, $n = 12$. Experiments were performed on two different experimental days. Statistically significant differences are denoted by $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.0001$, as determined by Student's *t*-test.

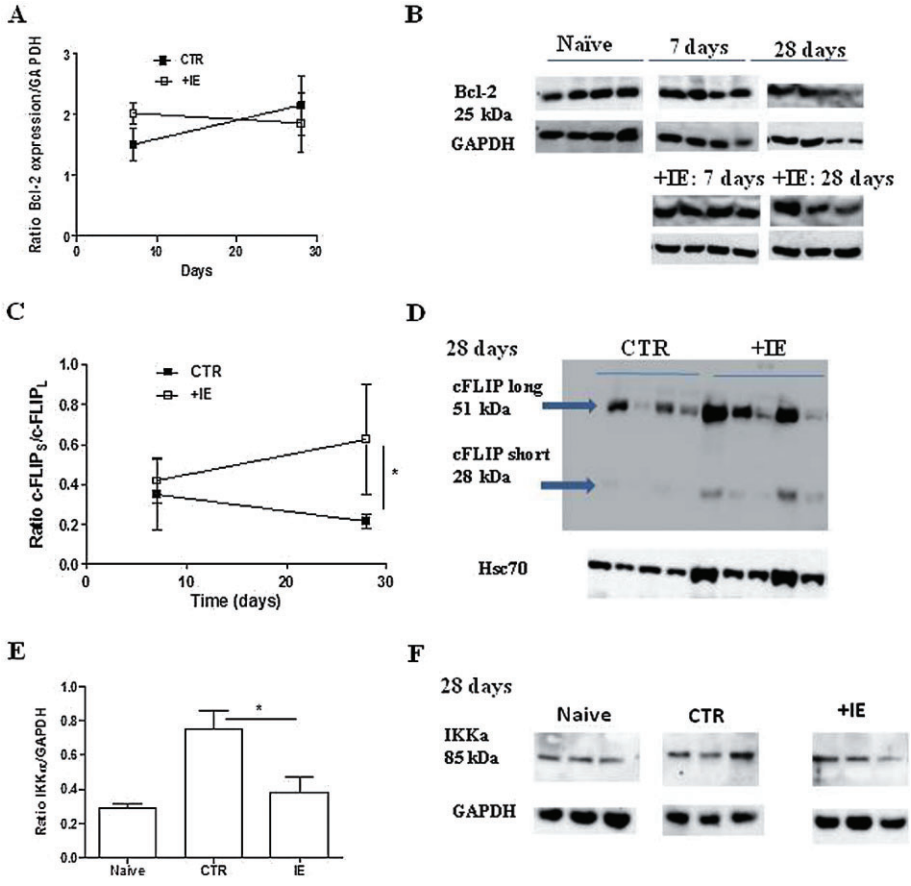


Figure 3

Involvement of caspase-8 in the apoptotic cascade. Lung tumour-bearing mice presented high levels of Bcl-2 (25 kDa) (A and B), which were not reduced after z-IETD-FMK (IE) treatment at either 7 or 28 days. In contrast, the levels of the short segment c-FLIPs (28 kDa) were significantly increased in the lung of tumour-bearing mice treated with z-IETD-FMK (C and D). The levels of IKKα were decreased in z-IETD-FMK-treated lungs of tumour-bearing mice compared with CTR mice (E and F). Western blot analyses were performed on lung homogenates and expressed as ratio between the target protein versus GAPDH or Hsc70 (loading control). Data represent mean ± SEM, $n = 7$. Experiments were performed on two different experimental days. Statistically significant differences are denoted by $^{*}P < 0.05$ as determined by Student's *t*-test.

the pro-inflammatory NF-κB-dependent signalling (Blander, 2014). NF-κB controls the transcription of many pro-inflammatory cytokines involved in inflammation-driven carcinogenesis and tumour progression (Terlizzi *et al.*, 2014). Indeed, we observed that the expression of IKKα was higher in CTR NMU-treated mice compared with naïve and z-IETD-FMK-treated mice (Figure 3E and F). To confirm this, the correlation between the activation of NF-κB and caspase-8 was also evaluated by measuring the release of NF-κB-dependent cytokines. The administration of z-IETD-FMK sig-

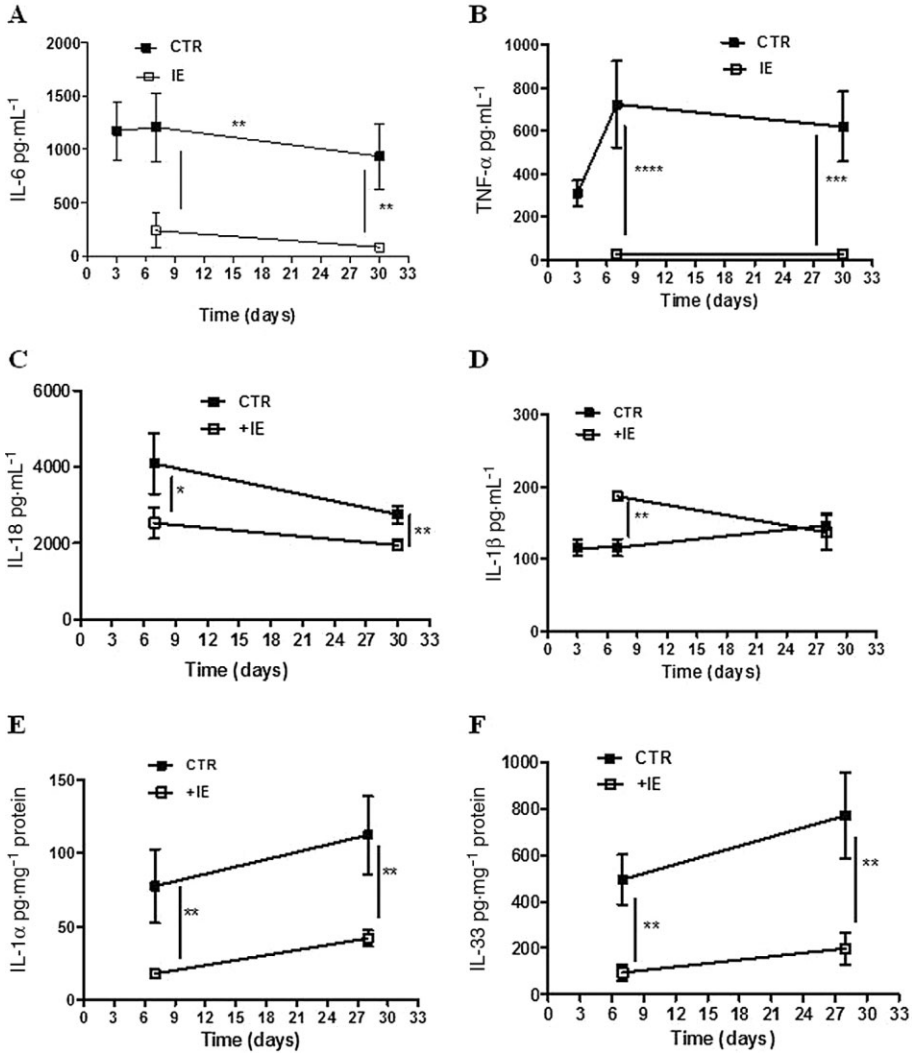


Figure 4

The pharmacological inhibition of caspase-8 reduces pro-inflammatory cytokine release. BAL fluid was harvested from lungs of tumour-bearing mice at both 7 and 28 days after NMU treatment. The pharmacological inhibition of caspase-8 by means of a specific inhibitor z-IETD-FMK (IE) significantly reduced the levels of IL-6 (A), TNF-α (B), IL-18 (C), but not of L-1β (D), in the BAL of lungs of tumour-bearing mice. Similarly, the levels of IL-1α (E) and IL-33 (F) were significantly reduced in lung homogenates obtained from control and z-IETD-FMK-treated tumour-bearing mice. Data represent mean ± SEM, $n = 12$. Experiments were performed on two different experimental days. Statistically significant differences are denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.0001$, as determined by two-way ANOVA with Bonferroni correction and Student's *t*-test.

nificantly reduced the levels of IL-6 (Figure 4A), TNF-α (Figure 4B), IL-18 (Figure 4C), but not IL-1β (Figure 4D), in the BAL obtained from lung tumour-bearing mice ($n = 12$) compared with the control group ($n = 12$). In addition, the levels of IL-1α (Figure 4E) and IL-33 (Figure 4F) were significantly lower in z-IETD-FMK-treated than in control lung homogenates obtained from tumour-bearing mice.

Taken together, our data show that caspase-8 is involved in the amplification of the NF-κB-dependent inflammatory process associated with lung cancer in mice. Furthermore, its pro-apoptotic activity was closely associated with the presence of the short segment of c-FLIP in both mice and humans.

Inhibition of caspase-8 modulates the innate immune microenvironment in the lung of tumour-bearing mice

Tumour immunoediting has been widely described as pivotal for the success of immunotherapy (Schreiber *et al.*, 2011). Therefore, we moved on to analyse the immune microenvironment in the lung of tumour-bearing mice treated with z-IETD-FMK. The percentage of macrophages (identified as $CD11c^{int}CD11b^{high}F4/80^{+}$) was reduced in z-IETD-FMK-treated tumour-bearing mice ($n = 12$) compared with control mice ($n = 12$) (Figure 5A). Similarly, myeloid-derived suppressor cells

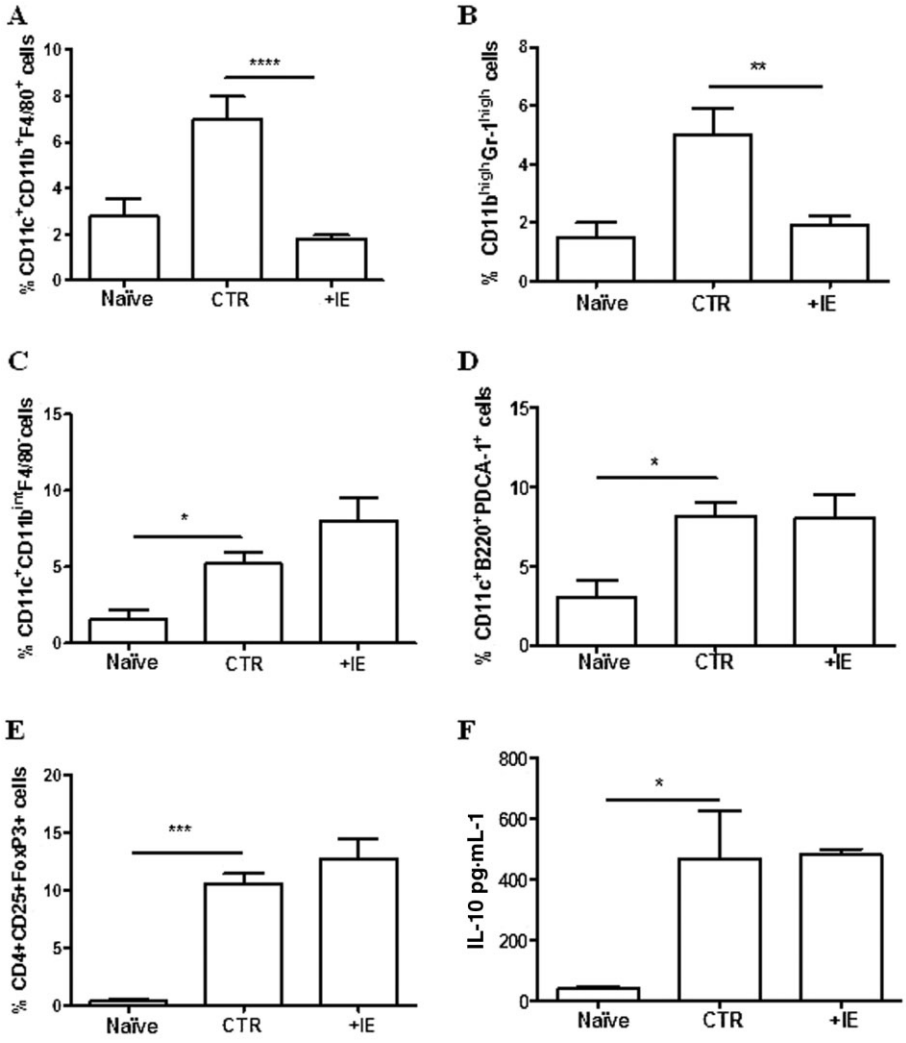


Figure 5

The pharmacological inhibition of caspase-8 modulates the immune microenvironment in the lung of tumour-bearing mice. The pharmacological inhibition of caspase-8 significantly reduced the percentage of macrophages (identified as CD11c ^{int}CD11b^{high}F4/80^{+} cells) (A) and MDSC (identified as CD11b^{high}Gr-1^{high} cells) (B), but not of dendritic cells (identified as CD11c^{high}CD11b^{int} cells) (C), plasmacytoid dendritic cells (identified as CD11cintB220+PDCA-1 + cells) (D) or T regulatory cells (identified as CD3+CD4+CD25+FoxP3+ cells) (E). (F) IL-10 levels in the BAL of CTR and z-IETD-FMK (IE)-treated lung tumour-bearing mice were not altered, but higher than in lungs of naïve mice. Data represent mean ± SEM, n = 12. Experiments were performed on two different experimental days. Statistically significant differences are denoted by *P < 0.05, **P < 0.01 and ****P < 0.0001, respectively, as determined by Student's t-test.

(MDSCs; identified as CD11b^{+}Gr-1^{high}) were significantly reduced in the lungs of tumour-bearing mice treated with z-IETD-FMK compared with controls (Figure 5B). In contrast, z-IETD-FMK treatment tended to increase the percentage of myeloid dendritic cell (DCs; identified as CD11c^{high}CD11b^{int}) compared with the control group (Figure 5C). However, these cells were not in their active form as we were not able to detect any differences in MHC I/II on the myeloid DCs from lungs of control or z-IETD-FMK-treated mice (data not shown). Moreover, the percentage of pDCs (identified as CD11c^{int}B220+PDCA-1+) (Figure 5D) and T regulatory cells (CD4+CD25+FoxP3+) (Figure 5E) did not show any statistical differences between control and z-IETD-FMK-treated mice. This latter effect was confirmed by higher levels of IL-10 in the BAL of both control and z-IETD-FMK-treated mice compared with naïve mice (Figure 5F).

Taken altogether, these data imply that although the pharmacological inhibition of caspase-8 diminishes the pro-inflammatory humoral pattern of the lung tumour microenvironment, it does not interfere with the recruitment of immune cells to the lung of tumour-bearing mice, implying that its activation does not play a role in the tumour-induced immunoediting.

Discussion

Recent evidence has demonstrated that besides its central role in apoptosis, caspase-8 can also modulate inflammation. Apoptosis and inflammation are two phenomena that, although in an inverse manner, are strictly correlated to tumour outgrowth. Human NSCLC cell lines have been shown to express high levels of pro-caspase-8 (Riley *et al.*, 2013). However, to our surprise, in the present study we showed that caspase-8 is in its active form in both healthy and cancerous lung tissues. In addition, by using a carcinogen-induced mouse model of lung cancer, we demonstrated that the inhibition of caspase-8 by means of z-IETD-FMK significantly reduced lung tumour burden and this was accompanied by lower pro-inflammatory cytokine release.

A common mechanism by which tumour cells evade the effects of pharmacological treatment is to alter the expression of the proteins involved in apoptosis and this paves the way for drug resistance (Juin *et al.*, 2013). Unlike SCLC, NSCLC, which represents 80% of lung cancer diagnosis, highly expresses the precursor and the active form of caspase-8 (Figure 1A). However, the pro-apoptotic activity of caspase-8 depends on the levels of the short segment of c-FLIP (Riley *et al.*, 2013; Blander, 2014). Indeed, cancerous tissues from lungs of both mice and humans did not express the short segment of c-FLIP; instead, the long segment was predominant. When the levels of c-FLIP$_L$ are high, pro-caspase-8 preferentially heterodimerizes with it leading to an insufficient amount of active caspase-8 to induce apoptosis, so favouring cell survival (Blander, 2014). Therefore, the limiting step for caspase-8-dependent pro-apoptotic activity is related to the presence of the short segment of c-FLIP. Healthy lung tissues had higher levels of c-FLIPs compared with the cancerous tissues, implying that the active form of caspase-8 in the tumour mass was not able to induce apoptosis but rather tumour cell proliferation. Indeed, the inhibition of caspase-8 by means of z-IETD-FMK reduced Ki-67-positive staining in the lung of tumour-bearing mice compared with the control group, implying that caspase-8 can facilitate tumour cell proliferation. In support of this, the short isoform of c-FLIP was evident only in mice treated with z-IETD-FMK but not in the control group. Lamkanfi *et al.* (2007) reported that the outcome of the pro-apoptotic activity of caspase-8 is most probably determined by the extent of its activation. Our data show that the active form of caspase-8 in both human and mouse samples was not pro-apoptotic, but instead amplified tumour-associated inflammation. The levels of IL-6, TNF-α, IL-18, IL-1α, IL-33, but not IL-1β, were significantly reduced in the lung of tumour-bearing mice treated with z-IETD-FMK. The release of these cytokines is associated with STAT-3 activation, a transcription factor able to induce malignant cell proliferation by up-regulating the expression of cell cycle regulators and of the anti-apoptotic genes (Terlizzi *et al.*, 2014). Indeed, the levels of the anti-apoptotic Bcl-2 were not altered in tumour-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 pro-apoptotic effectors caspase-3, caspase-9 and cytochrome c (data not shown). However, it should be noted that Bcl-2 levels were not modulated in the lung of tumour-bearing mice even after z-IETD-FMK treatment, implying that the inhibition of caspase-8 only alters the levels of inflammatory cytokines without affecting the apoptotic cascade.

Moreover, because NF-κB activation and the amplification of the NF-κB-IL-6/TNF-α-STAT-3 signalling cascade occur in most malignancies and facilitate the expression of pro-inflammatory and pro-survival genes (Hu *et al.*, 2013), and because it has been demonstrated that caspase-8 can orchestrate this pathway (Lamkanfi *et al.*, 2007; Blander, 2014; Gurung *et al.*, 2014), we demonstrated that the inhibition of caspase-8 decreased the levels of IKKα, which is closely associated with NF-κB activation. Therefore, we can speculate that a caspase-8-dependent non-canonical inflammasome may represent the switch for tumour-associated inflammation. The activation of the inflammasome leads to IL-1β release (Terlizzi *et al.*, 2014). In our conditions, we observed that IL-1β release was increased only at early time points after the inhibition of caspase-8, implying that the role of this cytokine is not crucial in our mouse model of lung cancer compared with 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 a low-rate survival from time of diagnosis (Dinarello, 2010; 2014). These cytokines, directly or via the induction of TNF-α and IL-6 (Vanden Berghe *et al.*, 2000), are involved in cell proliferation and survival (Fisher *et al.*, 2014), as well as cell adhesion and migration (Elinav *et al.*, 2013), all features of tumour progression and invasiveness. Pro-IL-1β, pro-IL-18 and pro-IL-33 are converted into their active form mainly by a caspase-1-dependent canonical inflammasome (Schroder and Tschopp, 2010). Although caspase-8 can substitute for caspase-1 and can function as an efficient IL-1-converting enzyme for proteolytic maturation of IL-1β (Schroder and Tschopp, 2010), we did not detect 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 cleavage by caspase-1. In models of diethylnitrosoamine-induced liver carcinoma (Jiang *et al.*, 2011), skin papillomas (Arwert *et al.*, 2010) and gastric carcinoma (Furuya, 2002), IL-1α is released by dying cells, which stimulate oxidative stress pathways, responsible for local inflammation and in some cases for cell rescue from death to provide tissue regeneration and subsequent accumulation of mutations leading to tumour initiation/progression (Rider *et al.*, 2013). In support of these findings, IL-1α-induced IL-6 activates STAT-3 and promotes liver as well as gastric tumorigenesis (Elinav *et al.*, 2013). Besides its role in tumour-associated inflammation, IL-1α activity is also associated with the activity of mutated K-Ras, one of the main oncogenes, that induces constitutive activation of NF-κB and AP-1, which on the one hand can promote an autocrine loop for further IL-1α expression/secretion, and on the other increase tumour burden (Tjomsland *et al.*, 2013). These processes were well characterized in a mouse model of pancreatic carcinoma.

Of note, the ability of caspase-8 to induce inflammasome in a non-canonical manner has also been demonstrated in cultured bone marrow-derived cells. To the best of our knowledge, our data are the first to demonstrate that the active form of caspase-8 is involved in lung carcinogenesis *in vivo* in

both humans and in mice. In addition, the pharmacological inhibition of caspase-8 was shown to have an anti-tumour effect and represents novel evidence not only for the involvement of caspase-8 in lung cancer but also for the involvement of the inflammasome in this context. Although the endogenous ligand responsible for caspase-8 activity in our model is still not known, we can conclude that caspase-8 is an important orchestrator of cancer-associated inflammation, and the presence of the short or long c-FLIP segment may represent the switch that determines whether caspase-8 induces tumour proliferation or tumour arrest/regression in the lung.

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Author contributions

M. T. and R. S.: conception, design and interpretation of data. M. T. and R. S.: acquisition of data and analyses. V. G. D. C., G. P. and A. G.: human sample surgery. M. T., V. G. D. C., G. P., A. G., A. P. and R. S.: drafting the article or revising it critically for important intellectual content.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Lung tumour incidence in a mouse model of carcinogen-induced lung cancer
Figure S2 Pharmacological inhibition of caspase-8 proved to have anti-tumour activity even after lung tumour formation.
Figure S3 Western blotting analyses showed that human cancerous samples (LC) of NSCLC patients were characterized by the presence of only the long isoform of c-FLIP (51 kDa) compared with the 'healthy' (H) counterpart which instead presented higher levels of the short segment of c-FLIP (28 kDa).